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TITLE: STAT5A Regulates the Survival of Mammary Epithelial Cells
and the Development of Mammary Cancer

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13. ABSTRACT (Maximum 200 Words) Members of the epidermal growth factor receptor (EGFR) family play a significant role in the initiation and progression of mammary epithelial cell transformation. EGFR stimulation can initiate mitogenic signaling through STAT proteins, particularly Stat5a and Stat3. Previously, we have reported that in the presence of an activated EGFR, deletion of Stat5a from the mammary epithelium, delayed mammary involution by 9 days, and hyperplasia and mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor and is involved in delaying mammary tumorigenesis. To evaluate the role of Stat5a and Stat3 in breast tumorigenesis, we examined Stat5a and Stat3 protein expression and activation in breast tumors derived from MMTV-Neu, MMTV-Py-V-MT and MMTV-int3 transgenic mice. We found that the tyrosine phosphorylation level of both Stat5a and Stat3 were elevated in MMTV-Neu breast tumors. To understand how the EGFR and its downstream kinase signaling pathway contributes to mammary epithelial cell transformation, we used the ErbB kinase inhibitor, AG1478; MAPK kinase (MEK) inhibitor, PD98059; Src kinase inhibitor, PP2; and Jak2/3 kinase inhibitor, AG490 in ErbB2-dependent BT-474, SKBR-3, and MDA-MB-231 human breast cancer cells. Treatment of these cell lines <i>in vitro</i> with the kinase inhibitors resulted in reversible G1 arrest in BT-474 and MDA-MB-231 cells. We are utilizing unique combinations of transgenic(MMTV-Neu) and knockout (Stat3 conditional KO and Stat5a null) mouse models to address the specific contribution of Stat5a and Stat3 in mammary epithelial transformation <i>in vivo</i> . Together these experiments will allow us to evaluate the contribution of these proteins in the initiation and progression of EGFR dependent mammary tumorigenesis.				
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Introduction

The objective of this research is to elucidate the relationship between the action of the transcription factor, Stat5a, and the regulation of programmed cell death in the mammary gland in order to establish the role of Stat5a in the development of breast cancer. The project is also interested in the possible mechanisms of compensation or competition for Stat5a action by related family member, Stat3. We achieved the objectives through the development of unique mouse models of breast cancer and the molecular analysis of the signaling pathways critical for the activation of the factors. The mouse models will permit evaluation of specific molecules involved in the Stat5a and Stat3 signaling pathway as modulators of involution and tumorigenesis.

Body

This research project began with interbreeding of the WAP-TGF α transgenic and Stat5a knockout mouse lines and to provide a detailed study of the effects of Stat5a deletion on WAP-TGF α induced tumor progression. This combination transgenic/knockout mouse line (TGF α /Stat5aKO) was established and was expanded through standard breeding protocols to generate sufficient experimental and control mice for the tumorigenesis studies. After 8 months of breeding and data collection it was evident that the absence of Stat5a did have an effect on the development of tumors in the WAP-TGF α transgenic tumor model. In the presence of TGF α overexpression, complete genetic deletion of Stat5a delayed initial mammary tumor development by 6 weeks. The alteration in the progression on involution was confirmed by histological analysis. WAP-TGF α transgenic mice have a significant delay in the onset of involution at the end of lactation. This delay is suggested to be one of the mechanisms for mammary tumor formation in this mouse model. It was observed that TGF α /Stat5aKO mammary glands underwent a significant reorganization and deletion of cell structure during involution. These changes replicated those observed during involution of a normal mammary gland. This observation suggested that the absence of Stat5a in the TGF α /Stat5aKO mice was allowing involution to occur and possibly reducing the tumorigenic capacity of the epithelium. Stat5a requires phosphorylation in order to be activated and is normally rapidly dephosphorylated at the beginning of involution (within 12 hours). Phosphorylation analyses of the tissues from WAP-TGF α transgenic mice revealed that Stat5a was inappropriately phosphorylated during involution. This provided evidence that the EGF signaling pathway was activating Stat5a during involution and preventing the regression of the epithelium. The EGF pathway was simultaneously activated implicating the EGF dependent signaling kinase, MAPK, as the molecule initiating this phosphorylation event. This confirmed our earlier suspicions about the potential action of TGF α on Stat5a signaling in the mammary gland. Detailed molecular analysis of proliferation and programmed cell death levels revealed that there was an increase in apoptosis in the TGF α /Stat5aKO mouse when compared to the WAP-TGF α transgenic control gland. Surprisingly, apoptosis was increased during late pregnancy in the TGF α /Stat5aKO glands when normally cell death is low or absent in the wild type gland. This was the first evidence that Stat5a can act as a survival factor for mammary epithelium. These experimental demonstrated the absence of Stat5a affected the initiation of involution and the levels of apoptosis in the WAP-TGF α mammary gland. This data lead us to conclude that Stat5a can mediate signaling from the EGF receptor and can influence the progression of EGF-initiated breast cancer.

The absence of Stat5a delayed the initial appearance of tumor formation by 6-8 weeks. It did not completely abrogate the appearance of tumors and the rate of tumor formation was the same as the control group. This suggested that the process of involution could remove significant numbers of cells that could potentially contribute to the development of tumors. But it also suggested that there remained sufficient cells after the process of involution that could respond to EGF-activated signaling, to initiate transformation of the epithelium. The data generated was written in "Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelium Cell Survival and Tumorigenesis", which had been published in Cell Growth and Differentiation in October 1999.

The effect of Stat5a on the level and expression of cell death regulating molecules was examined through RNase protection assay. No significant change in the expression patterns of in the Bcl-2 family members were observed. Experiments have been performed with specific kinase inhibitors to block the selected pathways that may phosphorylate and activate Stat5a protein. These experiments are aimed at determining the level and duration of exposure required for significant inhibition of Stat5a phosphorylation and possibly induction of Stat5a-dependent apoptosis. Implants were generated from plastic pellets combined with inhibitors for the MAPK, JAK and camp-dependent kinases. Results from these studies have demonstrated initial levels of kinase inhibitor required to effect minor morphological changes in the mammary epithelium immediately around implant. No change in Stat5a status has been detected. This data and the data published in the manuscript in October 1999, was presented in a platform talk at the keystone conference "Advances in Breast and Prostate Cancer" in Lake Tahoe in March of 2000.

A review article "Transforming growth factor alpha and mouse models of human breast cancer" was submitted and published in Oncogene in January 2000. This manuscript reviewed the known mouse models of human disease generated with the TGF α gene including the work with the TGF α /Stat5aKO.

This project is also interested in the possible mechanisms of compensation or competition for Stat5a action by related family members. Based on conclusions from our work with the TGF α /Stat5aKO mouse model a collaboration was setup with Dr, David Levys lab at New York Medical Center, New York to study to role of Stat3 in the development and transformation of the mammary gland.

Stat5a and Stat3 have reciprocal patterns of phosphorylation with levels of Stat5a decreasing and Stat3 increasing at the beginning of involution. These changes can be detected within 12 hours after weaning of the pups. In the mammary gland, Stat5 phosphorylation and activation can be induced by prolactin, EGF and growth hormone whereas the factors that activate Stat3 during involution are unknown,

but possible candidates include members of the IL-6 cytokine family, EGF, PDGF and IGF pathways. Since inactivation of the Stat3 gene results in embryonic lethality, the Cre-loxP recombination system had been used to inactivate this gene in mammary epithelium. The deletion of exon 22, which encodes a critical tyrosine residue, demonstrated a role for Stat3 in the regulation of the first phase of involution. Apoptosis of mammary epithelial cells was reduced during the first phase but involution was able to proceed after a delay of 3 days. In an attempt to further clarify and define the molecular roles of Stat3 in the process of mammary tissue remodeling we completely inactivated the Stat3 gene in mammary epithelium using a WAP-Cre transgene (WC) to excise exons 15-21 that had been bracketed by loxP sites. This region encodes 245 amino acids including the DNA-binding and SH2 domains. These experiments permitted us to address whether functional competence of mammary tissue upon weaning can be maintained in the absence of Stat3, and whether the protease-mediated second stage of involution depends on the presence of Stat3.

After tissue specific deletion of Stat3 in mouse mammary gland, we observed lack of epithelial remodeling in Stat3-null mammary tissue during involution stage and an apparent failure to cease secretion at a time that corresponded to the second, irreversible stage of involution. The alveolar structures retained a functional lactogenic phenotype. We put the pups back after 6 days of involution, the results demonstrated that the Stat3-null mammary glands were held in a functional stasis despite the loss of external lactogenic stimuli, and that lactation could resume after 6 days of involution. Histological analyses confirmed that Stat3-null mammary tissue displayed functional alveolar structures 5 days after the re-initiation of lactation, while control tissue did not display any secretory alveolar structures. Nuclear Stat5a was detected in the Stat3-null mammary epithelium after 6 days of involution followed by 5 days of lactation. Western blot analysis of these glands revealed that WAP levels had also increased after 5 days of suckling. These data demonstrate that the Stat3-null epithelium could reinitiate lactation after 6 days of forced involution, and display appropriate physiological markers. One of the hallmarks of mammary gland involution is the increase in programmed cell death and an increase in the expression of genes involved in the regulation of programmed cell death. Levels of TUNEL positive cells at day 2 or 6 of involution in the Stat3 null tissues were very low but not entirely absent. We conclude from this data that the program that initiates programmed cell death in the mammary gland is debilitated in the absence of Stat3. Since the loss of Stat3 prevented mammary tissue from being remodeled we hypothesized a delay in the activation of extracellular matrix metalloprotease (MMP) activity. We detected several differences in the pattern of protease activities between Stat3-null and control mammary tissue. MMP9 activity (92k) in Stat3-null samples during involution was delayed. In contrast, bands corresponding to the 72k proform and 62k active MMP2

did not show significant difference between the Stat3-null and control samples. The data generated was sufficient to write and submit a manuscript "Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli". The manuscript was accepted by Endocrinology in May, 2002.

To evaluate the role of Stat5a and Stat3 in breast tumorigenesis, we examined Stat5a and Stat3 protein expression and activation in breast tumors derived from MMTV-Neu, MMTV-Py-V-MT and MMTV-int3 transgenic mice. We found that the tyrosine phosphorylation level of both Stat5a and Stat3 were elevated in MMTV-Neu breast tumors when compared to MMTV-Py-V-MT and MMTV-int3 tumors. To understand how the EGFR and its downstream kinase signaling pathway contributes to mammary epithelial cell transformation, we used the ErbB kinase inhibitor, AG1478; MAPK kinase (MEK) inhibitor, PD98059; Src kinase inhibitor, PP2; and Jak2/3 kinase inhibitor, AG490 in ErbB2-dependent BT-474, SKBR-3, and MDA-MB-231 human breast cancer cells. Treatment of these cell lines *in vitro* with the kinase inhibitors resulted in reversible G1 arrest in BT-474 and MDA-MB-231 cells. We are utilizing unique combinations of transgenic and knockout mouse models to address the specific contribution of Stat5a and Stat3 in mammary epithelial transformation *in vivo*. We have bred conditional knockout Stat3 null and Stat5a null mice into mice expressing the MMTV-Neu transgene. These combination transgenic/knockout mouse lines (Neu/Stat3 conditional KO and Neu/Stat5aKO) were established and expanded through standard breeding protocols to generate sufficient experimental and control mice for the tumorigenesis studies. We have observed the mice for 10 months, no tumor developed in both experimental and control groups. We did histological analysis for 2 mice in each group. We found the Neu/Stat3 null mammary tissues were in hypercellular status when compared to the Neu transgenic mammary gland and wild type gland. Detailed molecular analysis of proliferation and programmed cell death levels revealed that there was an increase in proliferation in the TGF α /Stat3 null mouse when compared to the MMTV-Neu transgenic control and wild type glands. But there was no change in apoptosis between the experimental and control groups. We are observing the tumor progression in these mice. These data will be presented in a poster format to the Era of Hope Meeting at Orlando, Florida.

Together these experiments will allow us to evaluate the contribution of Stat5a and Stat3 in the initiation and progression of EGFR dependent mammary tumorigenesis. In addition, through the use of these specific kinase inhibitors, we hope to understand the specific STAT signaling pathways that mediate transformation of the breast. This work will allow development of directed and specific inhibitors of the kinases involved in breast cancer.

Key Research Accomplishments

1. **Paper:** *Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli.* The manuscript was accepted by Endocrinology in May, 2002.
2. **Poster:** Submitted to Era of Hope Meeting at Orlando, Florida. September, 2002.
3. Demonstrated that the tyrosine phosphorylation level of both Stat5a and Stat3 were higher in MMTV-Neu breast tumors.
4. Demonstrated that treatment with kinase inhibitors resulted in reversible G1 arrest in human breast cancer cell lines BT-474 and MDA-MB-231.
5. Demonstrated that the Neu/Stat3 null mammary tissues were in hypercellular status when compared to the Neu transgenic mammary gland and wild type gland. There was an increase in proliferation in the TGf α /Stat3 null mammary glands.
6. **Paper:** *Mammary Specific Deletion of Signal Transducer and Activator of Transcription 3 (STAT3) Delays Protease Activation.* Manuscript in preparation. Expected submission date August, 2001.
7. **Paper:** *Inhibition of Caspase Action Disrupts Ductal Outgrowth in the Mouse Mammary Gland* Manuscript in preparation. Expected submission date September 2001.
8. **Invited Seminar:** Roswell Park Cancer Institute, Department of Molecular and Cellular Biology, "STAT5a and Cancer" February 2001.
9. **Invited Seminar:** Pacific Northwest National Laboratory, "Inhibition of STAT Action and Development of The Mammary Gland." April 2001.
10. **Invited Seminar:** Roswell Park Cancer Institute, Department of Pharmacology and Therapeutics "Regulation of STAT Action and Mouse Models of Human Breast Cancer" July 2001.
11. **Invited Seminar:** Pharmacia Corporation, Kalamazoo MI, "STAT Regulation of Programmed Cell Death in the Mammary Gland" August, 2001.
12. **Invited Seminar:** Vanderbilt University Medical Center, Department of Medicine Nashville TN "STAT Regulation of Programmed Cell Death and Transformation in the Mammary Gland" August 2001.
13. **Poster:** Presented at Keystone Meeting "Cancer and Intervention", February 2001.
14. **Poster:** Presented at Breast Cancer Think Tank Meeting, July 2001. Received poster award to attend meeting.
15. Generated the MMTV-*neu*transgenic/STAT5a knockout mouse model of breast cancer.

16. In a collaborative effort with Dr. David Levy, generated the combined WAP-TGF α transgenic/ Stat3 knockout mouse model of breast cancer.
17. Demonstrated a connection between Stat5a-related molecule, STAT 3 and the progression of involution.
18. Established collaboration with Dr. Stephane Hunot to study the role of specific caspase knockout mice in normal mammary gland development.
19. Applied for numerous academic faculty positions based on research from this work. University of Colorado, Boulder; Roswell Park Cancer Institute, Buffalo; McGill University, Montreal; Dalhousie University, Halifax; Gonzaga University, Vanderbilt University, University of Southern Maine, University of Utah, National Cancer Institute, Frederick, University of California Davis, University of Colorado, Denver; University of Minnesota, Case Western University, University of Arizona, New Mexico State University. Applications were sent to several industrial opportunities.
20. **Paper:** *Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelial Cell Survival and Tumorigenesis* published in *Cell Growth and Differentiation* 10:685-694, October, 1999.
21. **Paper:** *Transforming growth factor alpha and mouse models of human breast cancer* published in *Oncogene* (review article) 19: 1085-1091, January ,2000.
22. **Platform talk :**Presented at Keystone Meeting "Advances in Breast and Prostate Cancer" , March , 2000.
23. **Poster:** Presented at Breast Cancer Think Tank Meeting, July, 1999. Received poster award to attend meeting.
24. Generated the combined WAP-TGF α transgenic Stat5a knockout mouse model of breast cancer.
25. Demonstrated Stat5a has a cell survival function in the mammary epithelium.
26. Demonstrated a connection between Stat5a activity and the development of mammary cancer.
27. Established that Stat5a has a regulatory role in the involution of the mammary gland .
28. Used an *in vivo* model of breast cancer to demonstrated that the regulation of the activity a transcription factor can affect the development and progression of transformation in the mammary gland.
29. Applied for several faculty positions based on research from this work. University of Washington, University of Arizona , University of Oregon, University of Washington (St. Louis).
30. Collected tissue from all animal models to generate a tissue bank of various stages of WAP-TGF α transgenic and TGF α /Stat5aKO mammary glands. These tissues were used for various mammary gland analyses within and outside our lab. Including microarray analysis of gene expression

of tumor types in the mammary gland. The data garnered from this analysis was used to prepare a manuscript from the Hennighausen lab on the development of a microarray analysis program(submitted).

31. Developed a collaboration to study the role of a related Stat molecule in the same breast cancer mouse model.
32. Received seven interviews based primarily on the research from my work and the training I received at the NIH. Two separate interviews in the Department of Molecular and Cellular Biology and in the Department of Pharmacology and Therapeutics at the Roswell Park Cancer Institute, Buffalo, New York. Additional interviews at Pacific Northwest National Laboratory in Kennewick Washington, Vanderbilt Medical Center Nashville TN and three industrial opportunities; Pharmacia, Kalamazoo, Michigan; Human Genome Sciences Rockville, Maryland and Protein Design Labs, Fremont California.

Reportable Outcomes

1. **Paper:** *Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli.* The manuscript was accepted by Endocrinology in May, 2002.
2. **Poster:** Submitted to Era of Hope Meeting at Orlando, Florida. September, 2002.
3. Generated the combined MMTV-Neu transgenic/Stat5a null mouse model and MMTV-Neu transgenic/ Stat3 conditional knockout mouse model of breast cancer.
4. **Invited Seminar:** Roswell Park Cancer Institute, Department of Molecular and Cellular Biology, "STAT5a and Cancer" February 2001.
5. **Invited Seminar:** Pacific Northwest National Laboratory, "Inhibition of STAT Action and Development of The Mammary Gland." April 2001.
6. **Invited Seminar:** Roswell Park Cancer Institute, Department of Pharmacology and Therapeutics "Regulation of STAT Action and Mouse Models of Human Breast Cancer" July 2001.
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13. **Platform talk:** Presented at Keystone Meeting "Advances in Breast and Prostate Cancer" , March , 2000.
14. **Poster:** Presented at Breast Cancer Think Tank Meeting, July, 1999. Received poster award to attend meeting.

15. Generated the combined WAP-TGF α transgenic/Stat5a knockout mouse model of breast cancer.

Conclusion

Stat5a is a survival factor, and its presence is required for the epithelium of the mammary gland to resist regression and involution-mediated apoptosis. Stat5a is one of the antecedents, locally acting molecules that initiate the process of epithelial regression and reorganization during involution. Tissue specific deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. The Neu/Stat3 conditional knockout mouse model will allow us to evaluate the contribution of Stat3 in the initiation and progression of EGFR dependent mammary tumorigenesis.

Deletion of Stat3 Blocks Mammary Gland Involution and Extends Functional Competence of the Secretory Epithelium in the Absence of Lactogenic Stimuli

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LOTHAR HENNIGHAUSEN

Laboratory of Genetics and Physiology (R.C.H., B.B., L.Z., L.H.), National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; New York University Medical Center (R.R., D.L.), Department of Pathology, New York, NY 10091

The transcription factor Stat3 is activated through tyrosine phosphorylation by many cytokines and is a fundamental mediator of their signals. In the mammary gland, Stat3 activity increases sharply shortly after weaning, and involution is delayed in mice, that contain a mutant Stat3 lacking 33 amino acids including the key tyrosine residue. We have now generated a more extensive mutation of Stat3 through the deletion of exons 15–21 in mammary epithelium. This resulted in the loss of 245 amino acids including the DNA binding and SH2 domains, and Stat3 protein was undetectable. Pregnancy-mediated mammary development and lactation were normal

in these mice. Involution was delayed and, remarkably, Stat3-null mammary epithelium maintained its functional integrity and competence even 6 d after weaning, whereas control mammary tissue was rendered nonfunctional within 2 d. The lack of remodeling and functional stasis of the epithelium correlated with the disruption of proteinase activity. Our data demonstrate that mammary tissue can retain its functional competence in the absence of external lactogenic stimuli and demonstrate a delay in the initiation of the irreversible stage of involution. (*Endocrinology* 143: 0000–0000, 2002)

EPITHELIAL CELL DEATH and remodeling of mammary tissue occur during involution and are regulated by changes in systemic hormones and local growth factors as well as interactions between the epithelium and the extracellular matrix (1, 2). The initiation of involution is characterized by changes in the activity of two members of the signal transducer and activators of transcription (STAT) family of transcription factors. The phosphorylation status, and thereby their activity, of the transcription factors Stat5 and Stat3 changes rapidly at the onset of involution (3). Stat5a and Stat3 have reciprocal patterns of phosphorylation with levels of Stat5a decreasing and Stat3 increasing at the beginning of involution. These changes can be detected within 12 h after weaning of the pups (3, 4). In the mammary gland, Stat5 phosphorylation and activation can be induced by prolactin (PRL), epidermal growth factor (EGF), and GH (5) whereas the factors that activate Stat3 during involution are unknown, but possible candidates include members of the IL-6 cytokine family, EGF, platelet-derived growth factor (PDGF), and IGF pathways (6–8).

The inactivation of Stat5 and Stat3 has demonstrated unique roles for these transcription factors in mammary physiology. Inactivation of the genes encoding Stat5a and 5b has revealed their essential role in the proliferation and differentiation of mammary alveolar epithelium during pregnancy (9, 10). In addition, Stat5a is a survival factor for mammary epithelium in the presence of a proliferative

growth stimulus (11). Because inactivation of the Stat3 gene results in embryonic lethality, the Cre-loxP recombination system had been used to inactivate this gene in mammary epithelium. The deletion of exon 22, which encodes a critical tyrosine residue, demonstrated a role for Stat3 in the regulation of the first phase of involution (12). Apoptosis of mammary epithelial cells was reduced during the first phase, but involution was able to proceed after a delay of 3 d. This result implicates that Stat3 may not play the dominant role in the regulation of all stages of involution and that additional factors control cell death and tissue remodeling. Alternatively, it is possible that this mutant Stat3, which lacked only 33 amino acids, retained some activity. In T cells, a mutant Stat3 with a loss of the region encoding the tyrosine residue at 705 possessed a dominant negative function (13).

In an attempt to further clarify and define the molecular roles of Stat3 in the process of mammary tissue remodeling, we completely inactivated the Stat3 gene in mammary epithelium using a WAP-Cre transgene (WC) to excise exons 15–21 that had been bracketed by loxP sites (14). This region encodes 245 amino acids including the DNA binding and SH2 domains. These experiments permitted us to address whether functional competence of mammary tissue upon weaning can be maintained in the absence of Stat3, and whether the protease-mediated second stage of involution depends on the presence of Stat3.

Materials and Methods

Materials

The generation of Stat5a antibodies has been described (3). Primary antibodies for antiphosphotyrosine and phosphatidylinositol 3-kinase

Abbreviations: EGF, Epidermal growth factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PRL, prolactin; STAT, signal transducer and activators of transcription; WC, WAP-Cre transgene.

AQ: D (PI3K) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Primary antibodies for β -catenin, actin, STAT3 (C-20), Bcl-x (S-18), Bax (N-20), STAT1 (E-23), SGP-2 (M-18) were purchased from Santa Cruz Biotechnology, Inc. (La Jolla, CA). Goat antirabbit and rabbit antimouse secondary antibodies were purchased from Transduction Laboratories, Inc. (Lexington, KY). Biotin labeled dUTP and terminal transferase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Super Signal Western Detection Kit was purchased from Pierce Chemical Co. (Rockford, IL).

Generation of STAT 3 fl/fl;WC

Creation of the mice that carry two Stat3 floxed alleles (Stat3 fl/fl) mice has been described previously (14). Briefly, a conditional allele of Stat3 was generated by homologous recombination with loxP sites inserted adjacent to exons 15 and 21 of the mouse Stat3 gene. A single male Stat3 fl/fl (SV129/C57BL6) founder was interbred with WAP-Cre (FvB/C57BL6) transgenic female mice to generate F1 founders heterozygous for the Stat3 floxed allele (Stat3 fl/+) and transgenic for WAP-Cre (Stat3 fl/+;WC). The resulting offspring were backcrossed five times to generate mice homozygous for Stat3 flox and transgenic for WAP-Cre. The same generation nontransgenic Stat3 fl/fl and nontransgenic Stat3 fl/+ littermates (Stat3) were used as controls for all molecular analyses. PCR confirmation of the presence of the Cre transgene and the Stat3 flox and null alleles has been described (14, 15). Genotype of the mice was confirmed after tissue collection by Western analysis for Stat3 expression. All animals were housed and handled according to the approved protocol established by the IUCAC and NIH guidelines.

Mammary gland collection

Mammary glands were surgically removed from anesthetized (and cervically dislocated if no further time points were needed) mice at d 10 of lactation and d 2, 6, and 9 of involution. Biopsies were performed on anesthetized (isoflurane gas) dams at the desired time point in a survival surgery conducted according to the approved protocol established by the IUCAC and NIH guidelines. The biopsies have not been found to alter the progression of involution in this or any prior studies using the surgical excision. Day 1 of involution was designated as 24 h after the morning that the pups were removed from the dam. Dams were allowed to lactate for 10 d, and then the pups were removed to induce involution. The mammary lymph node was removed before homogenization of all glands. Tissues were prepared immediately for RNA and protein extraction as described. Inguinal (number 4) mammary glands used for histological analysis were surgically excised, spread on Omniset tissue cages (Fisher Scientific, Pittsburgh, PA), fixed for 5 h in Tellyzinsky's fixative or overnight at 4 C in 10% neutral buffer formalin. Glands were stored in 70% ethanol until processed by standard embedding and sectioning techniques onto Probe-On Plus slides (Fisher Scientific). Sections were stained with standard hematoxylin and eosin.

Western analysis

Preparation of protein extracts for Western analysis has been described (3, 9, 11). Briefly, 2 mg of fresh and frozen tissue were homogenized in 2 ml of lysis buffer with protease inhibitors; phenylmethylsulfonyl fluoride, leupeptin, and aprotinin at 50 μ g/ml and the activated phosphotyrosyl-proteinase inhibitor sodium orthovanadate at 4 mM on ice. Protein lysates were rocked for 1 h at 4 C and then cleared by centrifugation at 14,000 \times g for 20 min. Protein concentration in the lysates was determined using Bio-Rad Laboratories, Inc. (Hercules, CA) Protein Assay Kit according to the manufacturer's protocol. Lysates containing 40 μ g of protein were mixed with 2 \times loading buffer and BME then heated to 90 C for 3 min. Samples were briefly spun, electrophoresed, under denaturing conditions, on 8%, 12%, or 14% precast Tris-glycine gels and transferred to polyvinylidene difluoride membranes according to the manufacturer's protocol (Novex, San Diego, CA). Western analysis was performed essentially as described with the following exceptions; primary antibody (dilution Stat5a 1:5000; Stat3 1:1000; Bax 1:1000; antiphosphotyrosine 1: 5000; Bcl-x 1:1000, PI3K 1:1000, WAP 1:5000, β -catenin 1:500, actin 1:5000) was incubated overnight at 4 C with gentle rocking and all incubations with antibodies and initial blocking was performed in 3% nonfat dried milk in 1 \times TBST. Detection was

performed with the Super Signal Western Detection Kit according to manufacturer's protocol (Pierce Chemical Co., Rockford, IL) and exposed to Kodak MR autoradiography film (Rochester, NY). Exposure times were between 1 sec and 2 min. Immunoprecipitations with anti-Stat5a and anti-Stat3 antibodies were carried out essentially as previously described (11). Stripping was performed by incubating blots for 25 min at room temperature in Antibody Stripping Solution from the Western Blot Recycling Kit according to the manufacturer's protocol (α Diagnostic, San Antonio, TX). Blots were washed in TBST and then blocked with 3% nonfat dried milk in TBST. Membranes were stripped and reprobed up to four successive times. A loading control gel was generated from a parallel SDS-PAGE gel that was fixed for 2 h in 50% methanol, 10% acetic acid, stained overnight in 50% methanol, 0.05% Brilliant Cresyl Blue. Gel was destained in 5% methanol, 7% acetic acid until the background cleared. Gel was dried according to the manufacturer's protocol (Research Products International, Mount Prospect, IL). The Western data shown are representative of separate analyses that have been performed in triplicate using different sample sets (12 Stat3-null and 6 control mice). Stat3 and Stat5a analyses have been performed on every blot to ensure deletion and compare the relative loading between blots.

Immunohistochemistry of STAT3 and STAT5a

Immunohistochemical detection of Stat3 and Stat5a has been described previously. Briefly, 4% neutral buffered formalin fixed, 5 μ m tissue sections were deparaffinized, incubated in tissue unmasking fluid (Vector Laboratories, Inc., Burlingame CA) and heated in a microwave for 2 min. Sections were allowed to cool for 10 min and then washed in PBS, blocked with 10% normal goat serum for 30 min at room temperature and then incubated overnight at 4 C with primary antibody at a dilution of 1:200 for Stat3 and 1:600 for Stat5a. A standard secondary antibody detection protocol with biotin avidin HRP antibodies identified the presence of antigen. Sections were counterstained with hematoxylin and dehydrated according to standard protocols.

Zymogram gel analysis

Coomassie stained gelatin zymogram SDS-PAGE was carried out using 40 μ g of protein from whole gland lysate essentially as described in the manufacturer's protocol (Novex/Invitrogen). Gelatin zymography was performed using unstained 10% gelatin embedded Tris-glycine gels from Invitrogen. After electrophoresis, renaturing, and developing the gelatin embedded Tris-glycine SDS-PAGE gels were fixed for 2 h in 50% methanol, 10% acetic acid, stained overnight in 50% methanol, 0.05% Brilliant Cresyl Blue. Gel was destained in 5% methanol, 7% acetic acid until the background cleared. Gel was dried according to the manufacturer's protocol (Research Products International, Mount Prospect, IL). Preparation and quantitation of protein lysates were described in the previous section describing the Western analysis. The image was captured in grayscale to demonstrate a clear contrast in loading and protease activity.

TUNEL assays

Protocols for TUNEL analysis have been previously described (11). Tissue sections from mammary glands analyzed for Stat3 deletion by Western analysis were used for the TUNEL analysis. Each apoptosis sample counted represents a minimum of three random fields (at \times 200 magnification) and a minimum of 1000 total cells per tissue section for each mouse. A minimum of three mice per time point for each genotype were collected and analyzed. We were able to use a Z-distribution for the statistical analysis of TUNEL positive cells with the data sets that included three mice each contributing 1000 individual units (cells counted), and a total of 3000 cells counted for each time point and genotype.

Results

Inactivation of the Stat3 gene in mammary epithelium

Stat3 fl/fl mice (14) were bred with WC mice (15) to generate Stat3 fl/fl;WC dams which were subsequently mated. The WAP-Cre transgene is highly active during pregnancy,

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and Cre-mediated recombination can be observed in the majority of mammary epithelial cells (16). The extent of excision of exons 15–21 and the loss of Stat3 protein were investigated by Southern and Western blot analyses, respectively. Genomic DNA was prepared using mammary tissue from Stat3 fl/+; WC and control mice at d 18 of pregnancy and d 8 of involution. The genomic DNA was analyzed for the presence of the wild-type, recombined and floxed alleles (Fig. 1A). At d 18 of pregnancy the recombined allele was predominant, suggesting efficient deletion of exons 15–21 of the Stat3 gene. The presence of an unrecombined allele is indicative of the presence of stroma, where the WAP-Cre gene is not active and the presence of the wild-type allele.

To verify the absence of Stat3 upon inactivation of the Stat3 gene we analyzed Stat3 on Western blots at d 10 of lactation, and d 2 and 6 after weaning (Fig. 1B). No Stat3 was detected in mammary tissue from Stat3 fl/fl;WC mice. In contrast Stat3 was detected in mammary tissue from wild-type mice at d 10 of lactation and increased levels at d 2 of involution (Fig. 1B). Stat3 is preferentially expressed in mammary epithelium and the stroma contributes little to the signal seen in normal mammary tissue. We also examined the expression of Stat1 and Stat5 that could potentially compensate for the absence of Stat3 (Fig. 1B). Stat5 levels decreased after weaning and Stat1 levels increased. The expression patterns of these STATs were similar in both Stat3 fl/fl;WC and control mice.

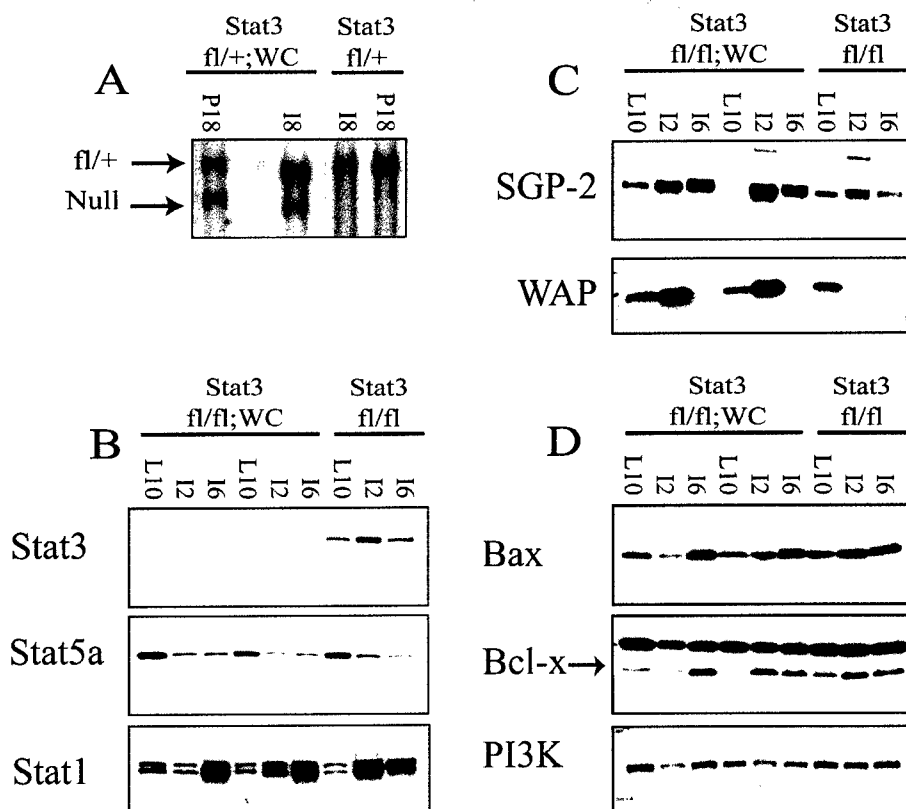
Expression of WAP and SGP-2 are maintained in Stat3-null mammary epithelium during involution

We examined the presence of milk proteins and other proteins linked to the maintenance and regression of mammary tissue. The milk protein WAP was detected in Stat3-null mammary tissue at d 10 of lactation followed by an increase at d 2 of involution (Fig. 1C). In contrast, WAP was not detected at d 2 of involution in wild-type controls. SGP-2 was detected during lactation in Stat3 fl/fl tissues, and its level was increased at d 2 of involution followed by a decrease at d 6 of involution (Fig. 1C). In contrast, SGP-2 levels remained high at d 6 of involution in Stat3 fl/fl;WC mammary tissue (Fig. 1C). We also examined the expression patterns of proteins linked to cell survival, cell death and cell integrity. The expression patterns of Bcl-x, Bax, and PI3K were comparable between control and Stat3-null mammary tissue (Fig. 1D).

Loss of Stat3 prolongs mammary involution

Stat3 fl/fl;WC and Stat3 fl/fl mice were mated and at d 10 of lactation the pups were removed. The integrity of mammary tissue was analyzed at d 10 of lactation and d 2, 6, and 9 of involution (Fig. 2). There was lack of epithelial remodeling in Stat3-null mammary tissue during these stages and an apparent failure to cease secretion at a time that corresponded to the second, irreversible stage of involution. The presence of lipid droplets and eosinophilic material within the alveoli at d 6 (Fig. 2F) suggested that the majority of

FIG. 1. Southern and Western blots demonstrating recombination of the Stat3 gene and Stat3 protein levels. DNA was analyzed from mammary tissue from d 18 of pregnancy and d 8 of involution from Stat3 fl/+;WC and Stat3 fl/+ mice (A). The floxed (fl/+) and recombined (null) alleles are shown. P18, pregnancy d 18; I8, involution d 8. The time points of P18 and I8 were selected to demonstrate the excision of the floxed exons before and after the points used for analysis. Proteins were analyzed at d 10 of lactation (L10) and d 2 (I2), and 6 (I6) of involution. A, Southern blot demonstrating recombination in fl/+ mice expressing WAP-Cre and fl/+ controls that do not express Cre. Separate mice were used for each time point with a total of four mice used for the Southern analysis. B, Western blot demonstrating the loss of Stat3 in mammary tissue from Stat3 fl/fl;WC mice. Stat5a levels decrease during involution in the absence and presence of Stat3. Stat1 is induced during involution in the absence and presence of Stat3. C, Western blot demonstrating differential expression of SGP-2 and WAP. D, Western blots showing the levels of Bax, Bcl-x, and PI3K.



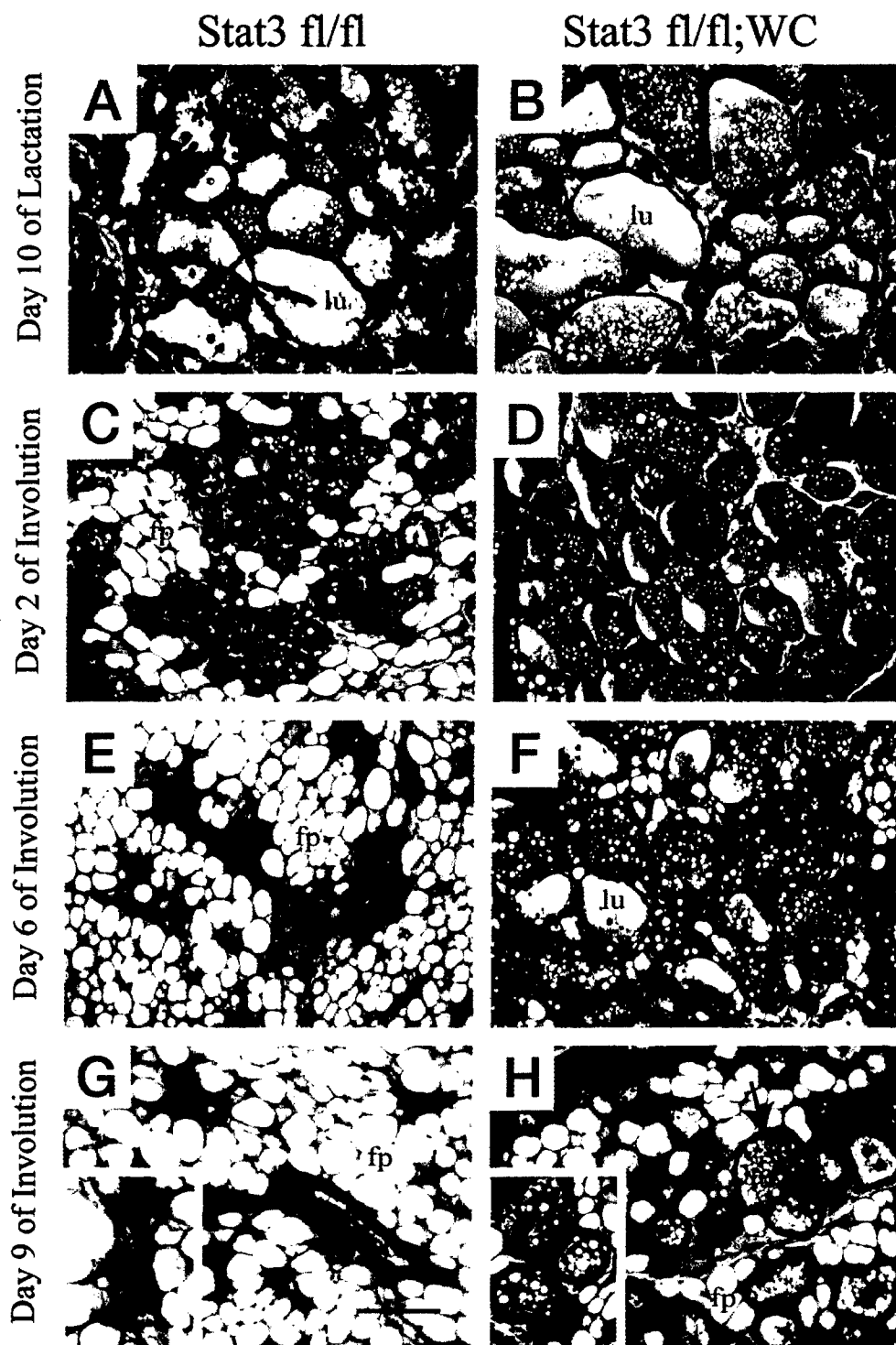


FIG. 2. Delayed involution of mammary epithelium in the absence of Stat3. Mammary tissue from Stat3 fl/fl (A, C, E, G) and Stat3 fl/fl;WC mice (B, D, F, H). At d 10 of lactation, there is very little difference between the Stat3 fl/fl and Stat3 fl/fl;WC tissues (A, B). The fat pad is filled and the alveoli formed lumina with lipid droplets. At d 2 of involution, the differences between Stat3 fl/fl and Stat3 fl/fl;WC tissues are visible (C, D). The fat cells reappeared with the collapse of alveoli in Stat3 fl/fl tissue whereas the Stat3 fl/fl;WC tissue retained its integrity. At d 6 of involution, Stat3 fl/fl mammary tissue has initiated extensive remodeling (E), whereas Stat3 fl/fl;WC tissue retained secretory alveoli that demonstrate a delay in the initiation of the second stage of involution (F). At d 9 of involution, Stat3 fl/fl tissue was similar to the virgin state, whereas secretory structures were retained in Stat3 fl/fl;WC tissue. Lipid droplets and expanded lumina were observed at d 9 of involution. Each inset is at $\times 2$ relative magnification to further illustrate the collapse of the Stat3 fl/fl lumen, whereas the Stat3 fl/fl;WC lumina remains expanded. The solid bar represents 200 μ m.

alveolar structures had retained a functional lactogenic phenotype. We did not observe any significant epithelial regression until d 14 after the initiation of involution (data not shown). The regression at d 14 of involution in the Stat3 null gland resembled the control samples at d 6 of involution. After 45 d of involution, the mutant glands were completely remodeled. Dams had no apparent difficulty nursing their pups during the second litter, and no significant difference was noted when the pregnancy occurred during the postpartum estrous. However, after their third pregnancy dams often displayed symptoms of mastitis and invasion of the mammary tissues by lymphocytes unless they had been allowed to involute for at least 3 wk before the third pregnancy. The symptoms of apparent infection and mastitis are similar to results observed in the previous Stat3 study (12).

Lactogenic competence is maintained upon weaning in the absence of Stat3

We hypothesized that, based on the apparent structural integrity, Stat3-null mammary tissue may have failed to enter the second irreversible phase of involution, and thus might be capable of reinitiating lactogenic function. To test this hypothesis, we investigated the functional state of Stat3-null glands after weaning. We allowed Stat3 fl/fl;WC and Stat3

fl/fl control mice to reach d 10 of lactation and removed the pups. After 6 d of involution, one no. 4 gland was surgically removed and 5-d-old pups were placed onto these dams. The pups were then allowed to suckle for 5 d. Ninety percent of the pups (18 of 20) placed onto the Stat3 fl/fl;WC dams survived. In contrast, all of the 12 pups placed onto the Stat3 fl/fl control dams died on or before 5 d of suckling. These results demonstrated that the Stat3-null mammary glands were held in a functional stasis despite the loss of external lactogenic stimuli, and that lactation could resume after 6 d of involution. Histological analyses confirmed that Stat3-null mammary tissue displayed functional alveolar structures 5 d after the reinitiation of lactation, whereas control tissue did not display any secretory alveolar structures (Fig. 3).

F3

Activation of Stat5 and WAP expression upon reinitiation of lactation in Stat3-null glands

A hallmark of lactation is the PRL-mediated activation and nuclear translocation of Stat5 in mammary epithelium. To establish whether Stat5 was activated in Stat3-null mammary epithelium that had gone through 6 d of involution followed by 5 d of suckling, we performed immunohistochemical analyses. Nuclear Stat5a was detected in the Stat3-null mammary epithelium after 6 d of involution followed by 5 d of

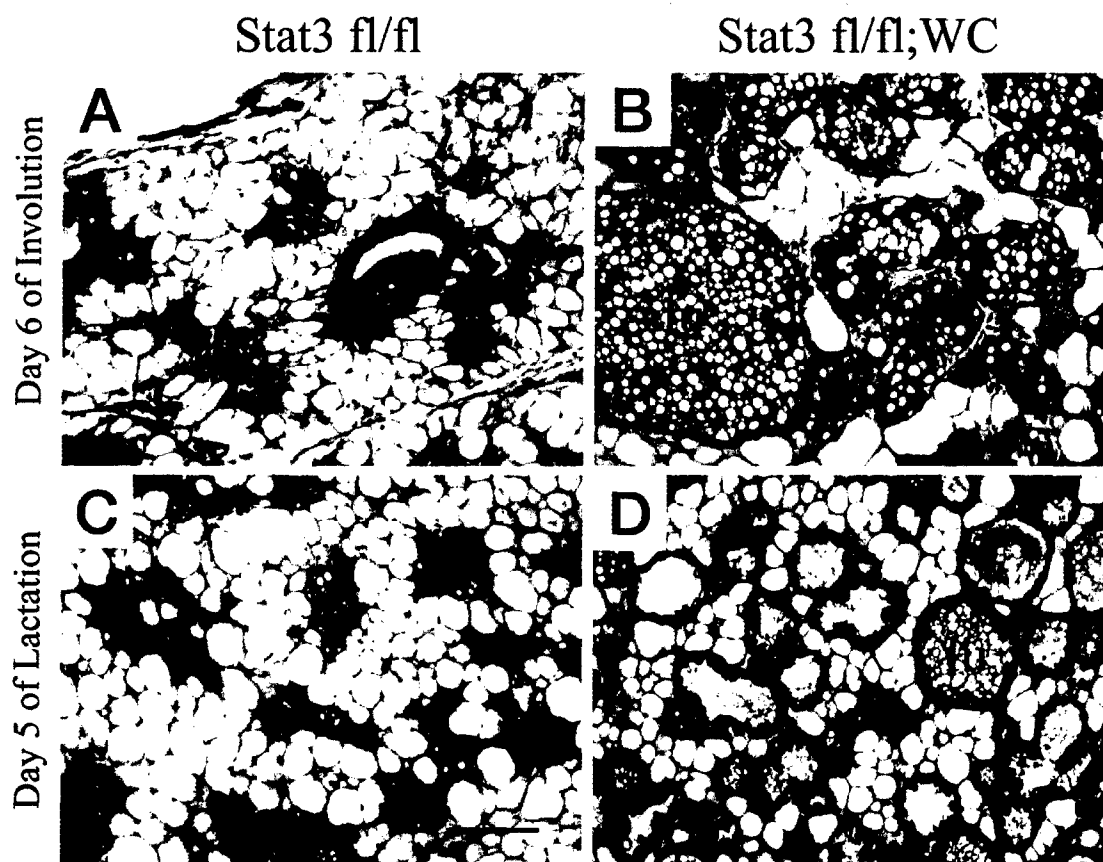


FIG. 3. Reestablishment of lactation in the absence of Stat3 after 6 d of involution. Mammary tissue from Stat3 fl/fl (A, C) and Stat3 fl/fl;WC mice (B, D). At d 6 of involution, Stat3 fl/fl mammary epithelium had been extensively remodeled, whereas Stat3 fl/fl;WC tissue had retained secretory features. Pups were placed with these dams, and after 5 d of suckling secretory features were seen in Stat3 fl/fl;WC but not Stat3 fl/fl mice. Stat3 fl/fl tissue did not respond to the external suckling stimulus. The solid bar represents 200 μ m.

F4 lactation (Fig. 4A, panel d). Western blot analysis of these glands revealed that WAP levels had also increased after 5 d of suckling (Fig. 4C). These data demonstrate that the Stat3-null epithelium could reinitiate lactation after 6 d of forced involution, and display appropriate physiological markers.

The absence of Stat3 in the mammary epithelium of Stat3 fl/fl;WC mice was confirmed by immunohistochemical analysis of sections from d 6 of involution and after 5 d of suckling. No nuclear Stat3 was observed in Stat3 fl/fl;WC mammary epithelium demonstrating that the gene had undergone Cre-mediated recombination (Fig. 4B, panels b and d). In contrast nuclear Stat3 was observed in Stat3 fl/fl mammary epithelium (Fig. 4B, panels a and c).

Reduced levels of apoptosis in the absence of Stat3

F5 One of the hallmarks of mammary gland involution is the increase in programmed cell death and an increase in the expression of genes involved in the regulation of programmed cell death (2). We examined the level of apoptosis in mammary tissue from Stat3 fl/fl;WC and Stat3 fl/fl control mice by TUNEL analysis (Fig. 5). The percentages of TUNEL positive cells were determined for Stat3 fl/fl;WC and Stat3 fl/fl tissues at 10 d of lactation and d 2 and d 6 of involution. The percentages obtained were 0.14 (\pm 0.03), 0.86 (\pm 0.03), and 1.16 (\pm 0.11) for Stat3 fl/fl;WC at 10 d of lactation, d 2 and d 6 of involution, respectively. The percentages obtained for the Stat3 fl/fl control tissues were 0.20 (\pm 0.10), 2.73 (\pm 0.19), and 2.52 (\pm 0.71) for 10 d of lactation, d 2 and d 6 of involution, respectively. The level of apoptosis was significantly reduced ($P < 0.05$) at d 2 of involution (significance determined using a Z-distribution $n=3$ mice with 1000 cells counted per mouse, and a total of 3000 cells counted at each time point for both Stat3 fl/fl and Stat3 fl/fl;WC using random fields for analysis). At 6 d of involution no significant difference could be determined within an appropriate statistical confidence interval between the Stat3 fl/fl and Stat3 fl/fl;WC tissues. Levels of TUNEL positive cells at d 2 or 6 of involution in the Stat3 null tissues were very low but not entirely absent. We conclude from this data that the program that initiates programmed cell death in the mammary gland is debilitated in the absence of Stat3.

Altered protease activity in the absence of Stat3

F6 A hallmark of involution of the mammary gland is the change in expression of proteases controlling the degradation of extracellular matrix, in particular the matrix metalloproteases (MMPs) and their inhibitors (17, 18). Because the loss of Stat3 prevented mammary tissue from being remodeled, we hypothesized a delay in the activation of extracellular MMP activity. To test this hypothesis, we investigated the pattern of protease activity using SDS-PAGE zymogram assays. Protein lysates from Stat3 fl/fl;WC and Stat3 fl/fl mammary tissue were separated in unstained gelatin embedded polyacrylamide gels, and the presence of protease activity was evaluated by the appearance of clear bands after Coomassie staining. We detected several differences in the pattern of protease activities between Stat3-null and control mammary tissue (Fig. 6). MMP9 activity (92k) in Stat3-null samples during involution was delayed (Fig. 6, arrow 2). In

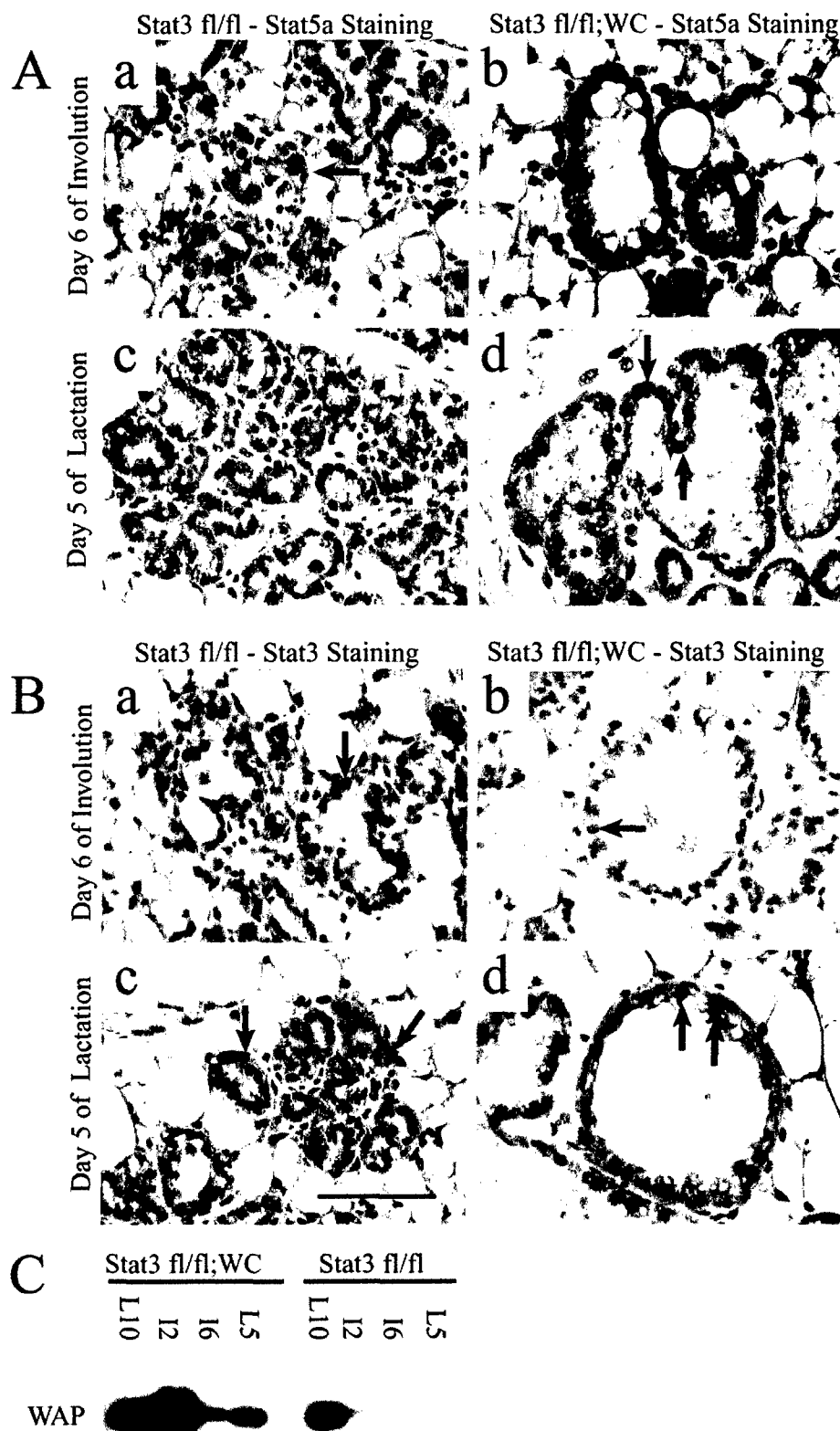
contrast, bands corresponding to the 72k proform and 62k active MMP2 did not show significant difference between the Stat3-null and control samples (Fig. 6, arrows 3 and 4, respectively).

Discussion

We have deleted exons 15–21 of the Stat3 gene in mammary epithelium during pregnancy using a WAP-Cre transgene that reliably recombined loxP sites located in different genes during pregnancy (15, 16, 19). The deleted sequence of 245 amino acids includes the DNA binding and SH2 domains. Although the excision generated an in-frame deletion, no truncated protein was detected, suggesting that it is unstable. The loss of Stat3 had profound effects on the progression of involution, both morphologically and functionally. There was little loss of epithelial cells and little remodeling in the first 6 d of involution, and even after 9 d secretory structures were evident. In contrast, remodeling was observed in the presence of Stat3 within 2 d after weaning. Notably, the Stat3 null mammary gland retained its structural integrity and functional competence even after 6 d of involution in the absence of external lactogenic stimuli. Importantly, lactation was reestablished in Stat3-null mammary epithelium after 6 d of involution, which was associated with an increase of nuclear Stat5a and WAP, hallmarks of active signaling through the PRL receptor. This argues that the effects of the loss of Stat3 on the mammary epithelium are dominant over changes that occur in response to systemic hormonal changes. It could be argued that the loss of Stat3 in cell types other than the mammary epithelium contributes to the delayed involution, possibly by changing the hormonal balance. However, this is unlikely as the WAP-Cre transgene has been used to delete several genes and tight mammary-specific recombination was observed in all cases. To completely rule out indirect influences, it was necessary to perform mammary transplantation experiments. We have transplanted mammary epithelium from Stat3 fl/fl;WC and Stat3 fl/fl;MMTV-Cre mice into wild-type hosts and investigated pregnancy-induced mammary development, which appeared to be normal in both cases (our unpublished data). **AQ:L** Because transplanted tissue does not have a connection to the nipple, it undergoes rapid remodeling after parturition and involution studies could not be performed at the time points needed for a direct comparison.

Our study confirms and extends previous research on the role of Stat3 in mammary gland involution (12). Although both studies point to a critical role of Stat3 in the process of involution, there are distinct differences between the observed phenotypes. We demonstrate that mammary tissue retained functional competence, whereas in the previous study the deletion of 33 amino acids resulted in a delay of involution and no functional rescue was reported. The differences could be the result of the targeting strategy and the level of Cre-mediated excision of Stat3. Although the previous study generated a Stat3 that left the DNA binding and SH2 domains intact, no Stat3 protein was detected in our study. The truncated Stat3 protein used by Chapman (12) has been associated with a dominant negative effect in T cells

FIG. 4. Stat5 and Stat3 activity after reestablishment of lactation. Dams were allowed to lactate for 10 d then the pups were removed to initiate involution. A, Stat5a immunohistochemistry in tissues from d 6 of involution (a, b) and d 5 of suckling after 6 d of involution (c, d). a and c, Stat3 fl/fl tissue showing cytoplasmic and nuclear Stat5a at d 6 of involution and a more dominant cytoplasmic staining at d 5 of suckling. b and d, Stat3 fl/fl;WC tissue with an abundance of cytoplasmic Stat5a at d 6 of involution and an increase in nuclear Stat5a at d 5 of reestablished lactation. Arrows indicate nuclei. B, Stat3 immunohistochemistry in tissues from d 6 of involution (a, b) and d 5 of suckling after 6 d of involution (c, d). a and c, Stat3 fl/fl tissues demonstrating the presence of cytoplasmic and nuclear Stat3 protein. b and d, Stat3 fl/fl;WC tissue; Stat3 protein is below a detectable level in the cytoplasm and nucleus of the alveolar epithelium indicating the loss of Stat3. Arrows indicate nuclei. C, Western blot showing the reinduction of WAP in Stat3 fl/fl;WC tissue at d 5 of suckling after 6 d of involution. The tissues used for lactation d 10 were biopsied from the same donors used for the d 2 involution time points. The tissues used for d 6 of involution were biopsied from the same donors used for the reinitiated lactation time points. Biopsies were conducted by surgically removing one no. 4 gland for the initial time point and the contralateral gland was collected after the specified period of involution. Induction of WAP was observed in the Stat3 fl/fl;WC tissue. Solid bar represents 125 μ m.



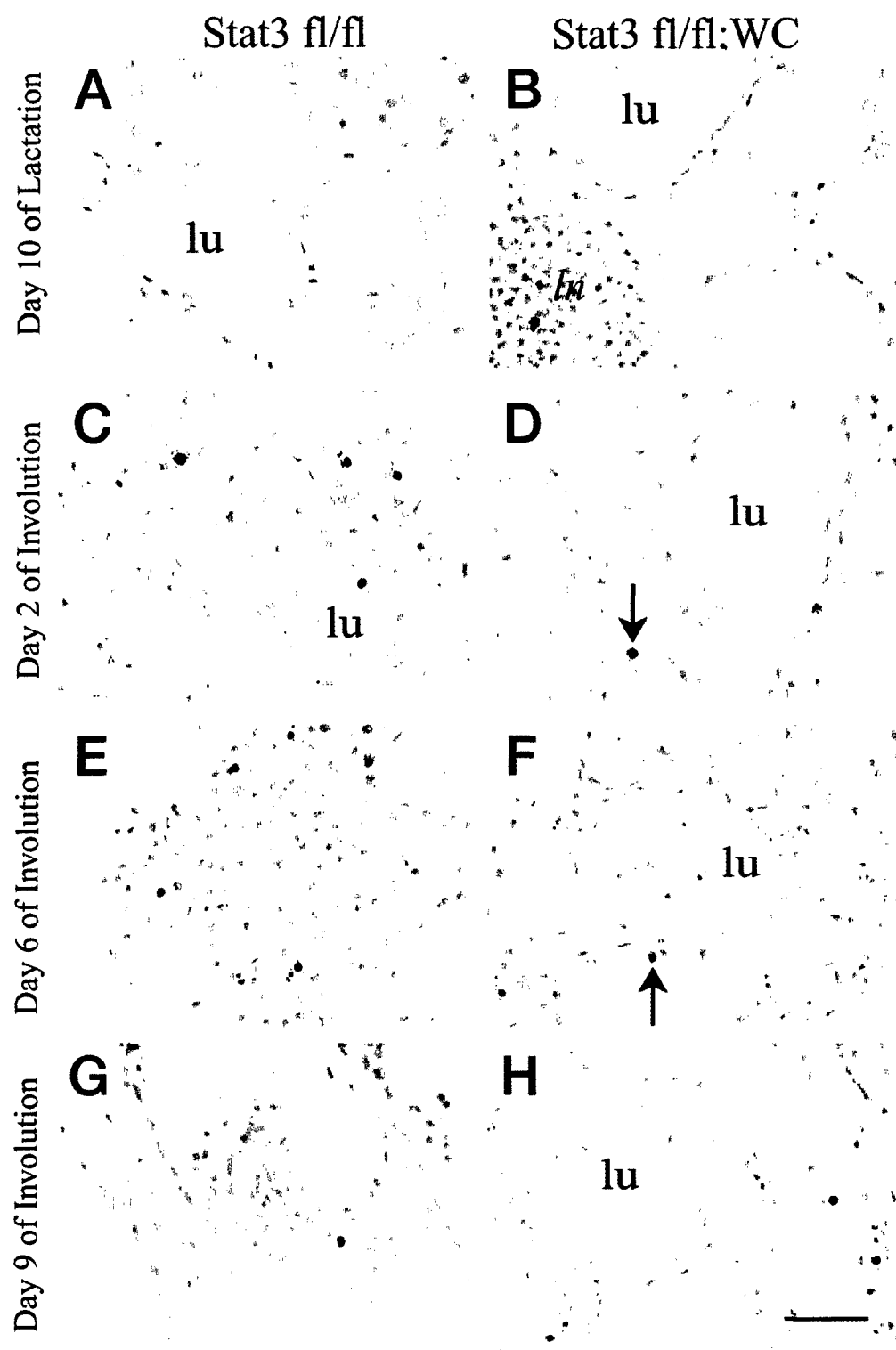


FIG. 5. Apoptosis in Stat3 fl/fl and Stat3 fl/fl;WC tissues. TUNEL staining was performed at d 10 of lactation and d 2, 6, and 9 of involution. Staining (A, B) at d 10 of lactation with no significant difference in apoptosis between the Stat3 fl/fl and Stat3 fl/fl;WC tissue. C, E, and G, d 2, 6, and 9 of involution from Stat3 fl/fl mammary tissue. D, F, and H, d 2, 6, and 9 of involution from Stat3 fl/fl;WC mammary tissue. There are fewer apoptotic cells present in the Stat3 fl/fl;WC tissue at 2 d of involution ($P < 0.05$). No statistically significant difference in the number of apoptotic cells could be determined for the other time points within an appropriate confidence interval. Many of the lumina in the Stat3 fl/fl;WC tissue remain fully expanded despite the occasional TUNEL positive apoptotic cells in near proximity. Solid bar represents 100 μ m. ln, Lymph node; lu, lumina.

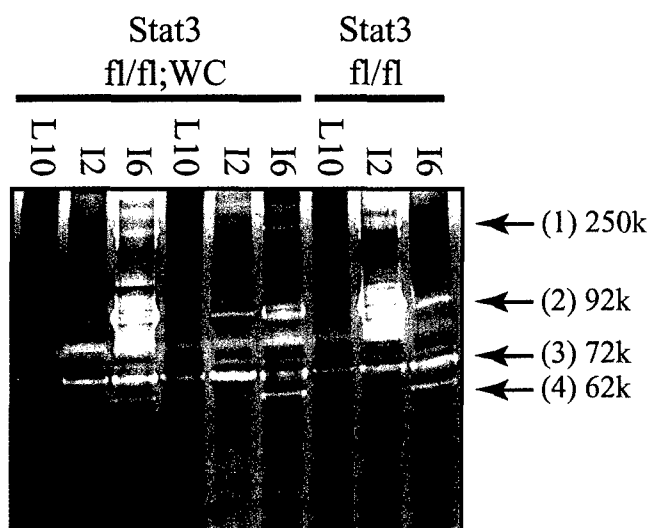


FIG. 6. Protease activity in Stat3 fl/fl and Stat3 fl/fl;WC tissues. Zymography was performed using 40 μ g of protein from whole tissue lysate on unstained gelatin embedded Tris-glycine sodium dodecyl sulfate gels. The gels were subsequently Coomassie stained to show both the protein loading and relative protease activity. The image shown is captured in grayscale to clearly define the contrast of the bands within the gel that demonstrate both loading and protease activity (1). An unknown gelatinase at 250K (2). MMP9 activity (92k) is delayed until d 6 of involution in Stat3 fl/fl;WC mice, whereas the activity in the Stat3 fl/fl controls is visible at d 2 (3, 4). MMP2 activity of the proform and active at 72k and 62k, respectively. The zymogram assays were repeated in triplicate with different sample sets and the data shown is representative of glands surgically removed at 10 d of lactation and collected at the specified point during forced involution. Two sets of Stat3 fl/fl;WC tissue are shown to illustrate the slight variance in expression of zymogen activity at d 6 of involution.

(13). Alternatively, it is possible that the Cre transgenes used in the two studies account for some of the differences.

Programmed cell death is induced in the mammary gland within 12 h of the end of suckling (20) and increases sharply during the first phase of involution. Alterations in the levels of programmed cell death have been demonstrated in other transgenic and knockout mouse models that elicit a disruption of involution (12, 21–24). We were able to report a significant decrease in the level of apoptosis at d 2. However, there was not a complete absence of programmed cell death at either d 2 or 6 of involution, implying that the regulation of apoptosis is not entirely dependent on the presence of Stat3. We were able to show an increase in the level and duration of SGP-2 expression, although it is a protein of unknown function with respect to direct biological significance during mammary development or involution. Many possible mechanisms and roles have been suggested for SGP-2; however, the expression of SGP-2 is not necessary for the apoptotic process to proceed (25). SGP-2 is a secreted protein that has been shown to adhere to cells near the point of secretion and may infer a cytoprotection against certain apoptotic stimuli (26–28). Cell survival of mammary epithelium at the interface of lactation and involution has been shown to depend on the presence of the Bcl-x protein (16). Because the Bcl-x levels were unaltered in the absence of Stat3, cell survival must depend on other proteins.

The second stage of involution is characterized by the

destruction of the lobuloalveolar unit and its surrounding extracellular matrix followed by a remodeling of the epithelial and stromal components (1, 29–31). MMPs are possible mediators of this process (32). A critical role for the MMPs and their inhibitors, tissue inhibitors of metalloproteinases, has been demonstrated in the development and maintenance of the mammary tissue (33, 34) and in the progression of involution (17, 22, 33). This function agrees with the disrupted protease pattern observed in the absence of Stat3. Therefore, we suggest that the loss of Stat3 causes a delay in the activity of the proteases and thereby alters the initiation and progression of the second irreversible stage during mammary involution. Stat3 and the IL6 family of cytokines that activate Stat3 can regulate the expression of MMP-3 and tissue inhibitor of metalloproteinase-1 (35, 36) *in vitro* and *in vivo*. However, the mechanism may be of indirect nature. Based on this and other studies (12), it is clear that functional loss of mammary epithelium after weaning requires the presence of Stat3, and we now show that Stat3 is also required for the activation of proteases *in vivo*. At this point, the molecular and mechanistic link between Stat3 and protease-mediated remodeling is not known.

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- B—Can Cre and loxP be defined here and in abbreviation footnote?
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- F—Location of Roche as meant?
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- H—Please write out BME.
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Transforming growth factor alpha and mouse models of human breast cancer

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Transforming growth factor alpha (TGF α) is a principal molecule in the normal and neoplastic development of the mammary gland. Binding of TGF α to the epidermal growth factor receptor (EGFR), activates the EGFRs' endogenous tyrosine kinase activity and stimulates growth of the epithelium in the virgin and pregnant mouse mammary gland. TGF α expression can be detected in breast cancer cells *in vivo* and *in vitro* and overexpression can elicit partial transformation or immortalized human and rodent mammary epithelial cells. Despite evidence implicating TGF α in the development of mammary neoplasia, the actual mechanism of TGF α -induced transformation is unclear. Transgenic mouse models targeting heterologous TGF α to the mammary gland have established TGF α overexpression can induce hyperproliferation, hyperplasia and occasional carcinoma. These transgenic studies demonstrated a facilitating, proliferative role for TGF α in the development of neoplasia and implicated several oncogenes that can cooperate with TGF α to transform the mammary epithelium. From studies of EGFR signaling pathways, inhibitory and modulating agents such as anti-EGFR antibodies and specific kinases inhibitors have been used to block the action of this pathway and prevent the development of TGF α -induced neoplasia and tumor formation. Studies in Stat5a knockout mice have established that the JAK2/Stat5a pathway can facilitate the survival of the mammary epithelium and can impact the progression of TGF α -mandated mammary tumorigenesis. Together these experiments indicate that TGF α and the EGFR signaling pathway are potentially amenable to therapies for treatment of human breast disease. *Oncogene* (2000) 19, 1085–1091.

Keywords: TGF alpha; mammary gland; transformation; cancer; mouse models; transgenic

Introduction

Development and progression of breast cancer, like many other types of human cancer, is dependent on the progressive corruption and alteration of normal signaling pathways. Signaling mechanisms that transmit growth signals are a predominant target for carcinogenic and oncogenic alterations as they often confer a growth advantage to the pre-neoplastic cell. One of the primary growth factor receptors

involved in normal and neoplastic development of the mammary gland is the EGFR and its extended family of related receptors and peptide ligands (Table 1). EGFR and the ligand TGF α , have expression levels altered in the development of cancer in numerous issues. The related family of ligands that bind to these receptors; EGF, heregulin, amphiregulin, and cripto also display alterations in their expression and activity within the pre-neoplastic and transformed cell (for a review see (Dickson and Lippman, 1995; Gullick *et al.*, 1999; Schroeder and Lee, 1997)). Despite the well characterized role of the EGFR and TGF α in normal mammary growth signaling, in transformation of mammary epithelial cells *in vitro* and *in vivo* and their elevated expression in some breast cancers, the specific alterations of the EGFR signaling mechanism that lead to TGF α -initiated neoplasia are poorly understood. In an attempt to elucidate these alterations, several transgenic mouse models have been generated to explore the role of TGF α and EGFR in mammary neoplasia. This review will focus on TGF α and its role in transformation of the mammary gland. In addition, it will examine those studies that have attempted to identify the mechanisms of TGF α action in the mammary gland and those molecules that are capable of cooperatively interacting with TGF α to promote tumorigenesis.

TGF α and EGFR expression coincides with normal proliferation in vivo

TGF α is structurally and functionally similar to EGF; the peptides share a 42% identity and can elicit the same biological effects in cultured mammary epithelial cells and explants (Daniel and Silberstein, 1985; Salomon *et al.*, 1987; Vonderhaar, 1987). TGF α is often co-expressed with the EGFR and binding to the receptor activates the EGFRs' endogenous tyrosine kinase activity. Mammary epithelial cell lines can be stimulated to proliferate by TGF α (Smith *et al.*, 1989; Zajchowski and Sager, 1991) and it can act as an autocrine growth factor in normal and immortalized human mammary epithelial cells. TGF α autocrine and proliferative activity can be blocked in these cells by an anti-EGFR antibody (Bates *et al.*, 1990; Kenney *et al.*, 1993).

In normal mammary gland development, TGF α and EGF transcripts can be detected in the ductal and lobuloalveolar stages. The virgin mouse mammary gland expresses TGF α in the proliferative cap cells and the stromal fibroblasts around the neck of the terminal endbud, whereas EGF expression is localized

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Table 1

Ligand	Receptor
<i>EGF-Like</i>	
EGF	EGFR
TGF α	EGFR
Amphiregulin	EGFR
Epiregulin	EGFR ErbB-4
Betacellulin	EGFR ErbB-3 ErbB-4
Heparin binding-EGF	EGFR ErbB-3 ErbB-4
<i>Heregulin/neuregulins</i>	
Heregulin-1 (α and β)	ErbB-3 ErbB-4
Heregulin-2 (α and β)	ErbB-3 ErbB-4
Heregulin-3	?
Heregulin-4	ErbB-4
Glial growth factor	?
<i>Cripto</i>	
Cripto-1	? unique receptor and erbB4
Cripto-3	?

to the luminal ductal epithelium (Snedeker *et al.*, 1991). In the absence of ovarian steroids, exogenous TGF α and EGF can stimulate ductal growth of the mouse mammary epithelium suggesting that they can act independently of secondary signals (Snedeker *et al.*, 1991). EGF and TGF α mRNA is present in pregnant and lactating rat and human mammary glands and increases 2–3-fold over virgin levels at pregnancy (Liscia *et al.*, 1990). The EGFR is required for ductal development (Wiesen *et al.*, 1999) and can be detected in both the stromal and epithelial components of the virgin mammary gland (Coleman *et al.*, 1988). In the pregnant gland, a rapid increase is observed during midpregnancy precisely at the time of extensive cellular proliferation (Edery *et al.*, 1985). After pregnancy, the levels of EGFR decrease significantly. The coincident expression of TGF α and its receptor with the proliferative phases of mammary epithelial growth and TGF α 's direct mitogenic effect on epithelium *in vitro* and *in vivo* confirms the functional role for this signaling dyad in the development and proliferation of the mammary epithelium.

TGF α expression is associated with human breast cancer

TGF α is expressed and mitotically active in numerous breast cancer cell lines and has been directly implicated as a modulator of transformation *in vivo* (Borellini and Oka, 1989; Daniel and Silberstein, 1985; de Jong *et al.*, 1998a; Salomon *et al.*, 1984; Valverius *et al.*, 1989). In fact, the discovery of TGF α was based on its ability to transform retrovirally-infected cultured fibroblasts (Todaro *et al.*, 1980; de Larco and Todaro, 1978). Expression of TGF α has been identified in pleural effusions from normal mammary gland (Arteaga *et al.*, 1988), in invasive ductal carcinoma (Pilichowska *et al.*, 1997) and correlates with increased neo-angiogenesis (de Jong *et al.*, 1998b) in breast tumors. Carcinomas of the breast that have higher level expression of TGF α also express high levels of EGFR, implicating a functional role for the TGF α /EGFR autocrine loop in tumors (Umekita *et al.*, 1992). The correlation between levels of EGFR expression and neoplastic transformation *in vivo* is controversial (Gullick and Srinivasan, 1998). Robert-

son and colleagues demonstrated that 40–60% of neoplasias examined in one study displayed normal levels of EGFR expression (Robertson *et al.*, 1996). Only a small proportion of breast cancers has elevated levels of EGFR (Slamon *et al.*, 1987), or amplification of EGFR (Peters and Wolff, 1983). This data implies that alterations in EGFR ligands may be more influential on the activity of the EGFR than changes in the levels of receptor expression itself. Alternatively, the formation of heterodimers between EGFR and the other erbB family members may play a role in effecting differential activities in tumorigenesis of the breast.

TGF α can modulate cellular transformation in breast cancer cell lines. In the immortalized human breast cancer cell line, MCF-7, TGF α transfected cells that expressed EGFR were stimulated to grow in a colony forming assay (Ciardiello *et al.*, 1990). In a parallel study with fully transformed MCF-7 cells that lacked EGFR, TGF α transfection did not effect a positive growth advantage (Clarke *et al.*, 1989). Overexpression of TGF α in the immortalized mouse mammary cell line, NOG-8, generated anchorage independent growth but colonies failed to form tumors in nude mice (Shankar *et al.*, 1989). Therefore, expression of TGF α is associated with the ability to stimulate growth, while overexpression is allied with partial transformation *in vitro* and correlated with appearance of carcinomas *in vivo*.

TGF α transgenic mouse models of breast cancer

Mouse models have been developed to study the role of TGF α in the transformation and development of the mammary gland. Several promoters have been utilized to target TGF α both non-specifically and specifically to the mammary gland. These mouse models have established the importance of TGF α in the early stages of neoplastic development of the mammary gland.

Initial studies demonstrating the neoplasia-promoting activity of TGF α were described in mice expressing human TGF α under the control of the zinc-inducible metallothionein (MT) promoter (Jhappan *et al.*, 1990). Despite the fact that expression of the transgene was low in the mammary gland and was generally not inducible, these mice displayed increased cellular proliferation and delayed epithelial penetration of the stromal fatpad during ductal development. In a second parallel study, using the MT promoter directing the expression of rat TGF α , Sandgren and colleagues noticed that mice that had passed through multiple pregnancies developed hyperplastic nodules and dysplasia of the mammary epithelium (Sandgren *et al.*, 1990). Only one mouse developed a secretory adenocarcinoma. These studies demonstrated that there was an association between the *in vivo* expression of TGF α and the development of mammary hyperplasia.

A study directing TGF α expression specifically to the mammary gland with the mouse mammary tumor virus (MMTV) promoter demonstrated unequivocally the growth and neoplasia promoting activity of TGF α (Matsui *et al.*, 1990). Precocious alveolar development, hyperproliferation and hyperplasias were apparent in the mature virgin mouse. The alveolar hyperplasia was present in glands from mature virgin mice, but not in

immature virgin mice, suggesting a requirement for secondary inputs from systemic hormones for any TGF α -dependent hyperplasia to occur. Hyperplastic alveolar nodules and cysts were prominent in the multiparous animal and increased in hyperplastic and dysplastic character with the number of pregnancies. One multiparous mouse developed adenocarcinomas although none were metastatic. A second study was performed with the MMTV-TGF α mice that studied in greater detail the generation of hyperplasia and tumors (Halter *et al.*, 1992). At one year, 65% of multiparous and 45% of virgin mice displayed hyperplasia. By 16 months, 40% of the multiparous and 30% of the virgin mice and mice had generated tumors. These studies demonstrated that TGF α could stimulate the proliferation of the mammary epithelium and generate proliferative late pregnancy mammary gland phenotypes in early pregnant animals. In addition, the hyperplasia, dysplasia and frank carcinoma observed with this mouse model suggested that there was a multistage nature to the progression of TGF α -initiated transformation.

A more dramatic mammary phenotype was achieved by targeting rat TGF α exclusively to the mammary gland with the WAP-TGF α transgenic mouse (Sandgren *et al.*, 1995). This transgenic model achieved high level TGF α expression during pregnancy and lactation and displayed similar proliferative mammary gland phenotypes as described in the MT and MMTV transgenic models but with an increased incidence

and decreased latency of tumors. In addition, the process of involution, in which the gland absorbs and reorganizes a significant portion of its epithelial structure, was delayed in these mice as it was in the original MT mice (Sandgren *et al.*, 1990). Latency of tumor appearance was decreased in comparison to the MMTV-TGF α model, but still required several rounds of pregnancy for initial tumor development. A majority of the tumors were well-differentiated glandular adenomas or carcinomas (53%) and fibroadenomas (35%) and retained the glandular characteristics of late pregnant mammary epithelium. The appearance of multiple types of tumor and the retention of normal glandular morphologies suggested that these different types were stages of TGF α -dependent tumor progression. Interestingly, the levels of cyclinD1 were elevated in the WAP-TGF α transgenic glands. The targeted overexpression of this cell cycle regulatory molecule is known to effect mammary transformation (Wang *et al.*, 1994) and is in a chromosomal region frequently rearranged in human breast cancer (Dickson *et al.*, 1995; Gillett *et al.*, 1994, 1996, 1999). This indicates that cyclinD1 may be a cooperative factor in the development of TGF α induced mammary carcinogenesis. The delay in involution was suggested as a mechanism for promoting transformation of the mammary gland as it provided an expanded population of proliferative epithelial cells that could be predisposed to transformation. This seminal paper demonstrated TGF α could transform the mammary

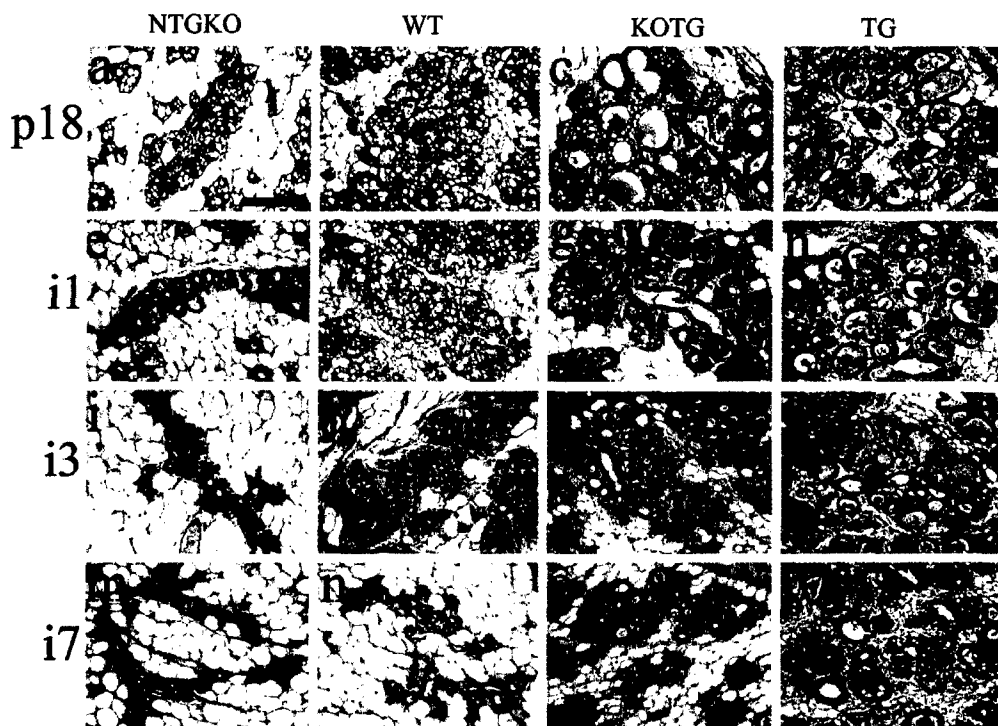


Figure 1 Involution is enhanced in the absence of Stat5a. Hematoxylin and eosin staining of inguinal mammary glands from Stat5a null non-transgenic (NTGKO), wildtype (WT), Stat5a null TGF α transgenic (KOTG) and TGF α transgenic (TG) mice at 18 days of pregnancy (p18/a-d), days 1 (i1/e-h), 3 (i3/i-l) and 7 (i7/m-p) of involution. Samples were collected from mice in or immediately after their first pregnancy. Compare epithelial condensation at day 3 and 7 of involution in the wildtype (i-j) versus the KOTG (i-k) and TG (i-l). Magnification is defined by the bar in a = 200 μ m. Reproduced with permission (Humphreys and Hennighausen, 1999)

epithelium and suggested a possible mechanism for neoplastic development. The observation that TGF α could cause a delay in involution was supported by results from a separate study with MT-TGF α mice (Smith *et al.*, 1995). An increase in DNA synthesis in lactation was accompanied by a significant decrease in apoptotic cells after 2 days of involution. Involution was also affected in a mouse model utilizing WAP-TGF α and the Stat5a knockout mouse (Humphreys and Hennighausen, 1999). In the absence of Stat5a, apoptosis levels rose in the mammary gland during pregnancy and involution. This increase in apoptosis enhanced involution of the WAP-TGF α gland (Figure 1) and increased the latency of WAP-TGF α -induced tumors. This result demonstrated the WAP-TGF α -induced delay in involution and tumor formation, could be abrogated by a downstream signaling molecule that regulates cell death in the mammary epithelium.

Subsequent studies examined the role that oncogenes may play in acting cooperatively with TGF α to promote mammary transformation. The oncogene *c-myc* is overexpressed in 25–30% of breast cancer (Bonilla *et al.*, 1988; Callahan and Campbell, 1989; Mariani-Costantini *et al.*, 1988, 1989; Morse *et al.*,

1988) and in rodent mammary epithelial cell lines transfected with *myc*, TGF α could cooperate to support a transformed phenotype (Telang *et al.*, 1990). Mice that overexpressed both *c-myc* and TGF α in the mammary gland had an increased tumor incidence and decreased tumor latency when compared to *c-myc* transgenics (Amundadottir *et al.*, 1995). A second paper utilizing the same bitransgenic mice demonstrated that the synergism between these two proteins was due to a cooperative growth stimulus and inhibition of *c-myc*-induced apoptosis by TGF α (Amundadottir *et al.*, 1996). Apoptotic tumors were present exclusively in mice that expressed only *c-myc*. In cell lines derived from these tumors, exogenous TGF α could inhibit apoptosis. These data again implicated TGF α as a survival factor in the mammary epithelium. Interestingly, tumor cells from the mammary glands of these bitransgenic mice could only become apoptotic when exposed to a specific inhibitor of the EGFR kinase pathway. This result suggested that an intact TGF α /EGFR autocrine loop was required to mediate the survival effects of TGF α .

Interaction between TGF α and the proto-oncogene *neu* was examined in bitransgenic mice expressing both

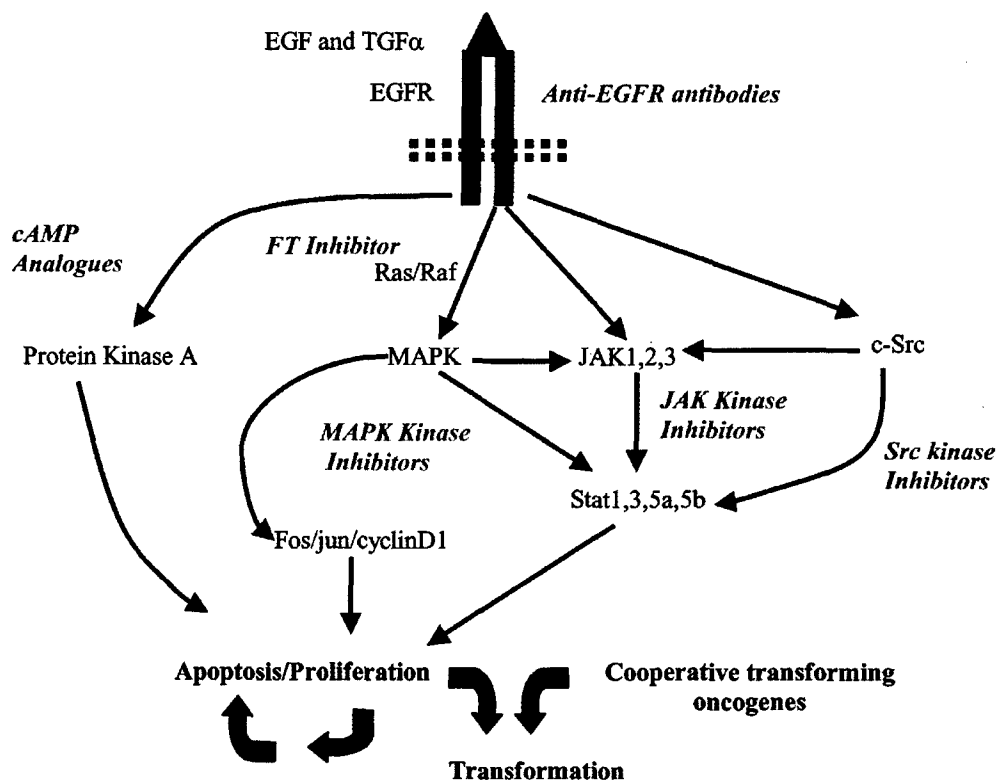


Figure 2 Model of the TGF α /EGF signal transduction pathway and EGFR signaling inhibitors that affect EGFR-mediated transformation. EGF and TGF α binding to the EGFR stimulate the activation of the endogenous receptor tyrosine kinase. The membrane-bound EGFR kinase activates one of several intracellular signal transduction pathways including the protein kinase A, Ras/Raf/MAPK, *c-src* and Jak/Stat pathways. Direct or indirect phosphorylation and activation of one or more of the Stat proteins, 1, 3, 5a, and 5b can be achieved through several of these mechanisms. The activating kinase, attributes of the targeted Stat and phosphorylated residue can elicit distinct functional consequences for the cell. Multiple intercellular pathways can lead to activation of the Stats and other nuclear factors like *myc* and cyclinD1. Each of these pathways can be blocked by specific inhibitors (shown in red Italics). The proliferative stimulus provided by TGF α and EGF in cooperation with transforming oncogenes can lead to cellular transformation. Consequently, a clear understanding of the mechanisms involved and the use of multifocal inhibitors of signaling intermediaries is critical for effectual inhibition of EGFR signal transduction. Some alternative and intermediary intercellular signaling molecules have been omitted for clarity

genes under the control of the MMTV promoter (Muller *et al.*, 1996). The *neu* proto-oncogene is a member of the EGFR family and although it does not bind to TGF α or EGF, it can form heterodimers with the EGFR and it is often found overexpressed and amplified in human breast cancers (Slamon *et al.*, 1987). Tumor latency was decreased in the bitransgenic mice, compared to the TGF α and *neu*, mono-transgenic lines. At 150 days 95% of the bitransgenic mice had mammary tumors vs 6% and 35% for the TGF α and *neu* mice, respectively. Bitransgenic tumors were multifocal whereas mono-transgenic lines generated focal tumors and contained activated *neu*. This paper suggested a novel mechanism for TGF α cooperativity involving the transactivation of *neu* through the EGFR. Treatment of MMTV-TGF α mice with the tumor promoter 7, 12 dimethyl benzanthrane demonstrated that TGF α could accelerate tumor formation and the authors suggested that TGF α could act as a tumor promoter (Coffey *et al.*, 1994).

These studies on the mechanism of TGF α -mediated tumorigenesis and cooperativity in the mammary gland demonstrate that the overexpression of TGF α gives the preneoplastic mammary epithelium a proliferative advantage but in and of itself is not a transforming event. Secondary events, like the activation of proto-oncogenes, can significantly increase the efficiency with which TGF α can transform the mammary epithelium. Importantly, these studies support the theory that TGF α 's influence on the regulation of apoptosis in the mammary gland is a possible mechanism of promoting survival of the neoplastic cell.

Disruption of TGF α signaling and potential therapies for breast cancer

The EGFR is required for mammary gland ductal development (Wiesen *et al.*, 1999; Xie *et al.*, 1997) and, as described previously, is aberrantly expressed in 40% of human breast cancers and occasionally overexpressed in those tumors with poor prognosis. This receptor can interact with several distinct ligands (Table 1) and activates different intracellular and nuclear signaling pathways (Figure 2). Substrates for this type 1 receptor tyrosine kinase are numerous and demonstrate the influence of EGFR on the regulation of cellular growth. Importantly, it is clear that this receptor plays a central role in the development and progression of human breast cancer. Therefore, insight into EGFR signal transduction and the mechanism of substrate selection and activation are critical to understanding the development of breast cancer. The EGFR and its interaction with TGF α has been and continues to be a target for potential anti-cancer therapies that aim to regulate its activity and signaling mechanisms.

A recent study demonstrated that inhibition of the signal from EGFR to its intracellular signaling molecules is critical in blocking tumor progression. Treatment of MMTV-TGF α transgenic mice with a farnesyl transferase inhibitor, which blocks Ras function, demonstrated a significant regression of mammary tumors (Norgaard *et al.*, 1999). This inhibitory effect was ineffectual after tumor accelera-

tion from multipregnancy or after treatment with the carcinogen DMBA supporting the theory that that EGFR signaling is important in the initial stage of neoplastic development. The EGFR can activate the intracellular tyrosine kinases Jak1 and the transcription factors Stat1, 3 and 5b in response to growth signaling from EGF (Leonard and O'Shea, 1998). Recent data has demonstrated that growth hormone activated Ras/Raf/MAPK pathway can directly activate the normally cytokine-activated pathway of Stat5a (Pircher *et al.*, 1999) and EGF can phosphorylate Stat5a through *c-src* (Olayioye *et al.*, 1999). Stat5a, a prolactin-activated transcription factor, has an established role in the development and differentiation of the lactation-competent mammary gland (Liu *et al.*, 1987) but is also associated with signaling in tumor cells (Hayakawa *et al.*, 1998; Richer *et al.*, 1998; Yu *et al.*, 1997; Zhang *et al.*, 1996). Stat5a-null mice interbred with WAP-TGF α transgenic mice revealed inhibition of TGF α -dependent MAPK activation and TGF α -dependent apoptosis inhibition after deletion of Stat5a. The increase in apoptosis permitted a more complete epithelial regression to occur at involution (Figure 1). A more complete regression deleted a significant number of potentially neoplastic cells and this impacted WAP-TGF α driven tumorigenesis. Stat5a null/WAP TGF α transgenic mice had an increase in tumor latency when compared to the WAP-TGF α transgenic. These mice displayed an increase in apoptosis before and during involution. These data suggested Stat5a was acting as a survival factor for the mammary epithelium by blocking the onset of apoptosis. Theoretically, the absence of Stat5a permitted apoptosis to occur and thereby diminished the pool of potential neoplastic cells that could become transformed (Humphreys and Hennighausen, 1999).

A novel mechanism of inhibition of TGF α activity was demonstrated recently in human mammary epithelial cells with an anti-metalloproteinase. Metalloproteinases are extracellular enzymes that cleave components of the extracellular matrix including the EGFR ligands; TGF α and EGF. This cleavage event releases them from the cell surface rendering the growth factor into an active form. These metalloproteinase inhibitors prevented TGF α release from the cell surface, blocked cell migration and decreased proliferation. Additionally, the metalloproteinase inhibitors reduced the growth of EGF-dependent tumor cell lines and could synergize with anti-EGFR antibodies (Dong *et al.*, 1999).

Therapies for inhibiting the action of EGFR and indirectly the action of TGF α , to block tumorigenesis are being explored. Current research into the action of molecules that may have a role in regulating the TGF α signaling pathway like EGFR-specific monoclonal antibodies (Ciardiello *et al.*, 1999), protein kinase A inhibitors, a combination of antibodies and chemotherapeutic agents (Bianco *et al.*, 1997; Ciardiello *et al.*, 1996; Ciardiello and Tortora, 1998), anti-estrogens like taxol, raloxifen, alone or in combination with antibodies like the humanized monoclonal antibody to *neu*; Herceptin (Brenner and Adams, 1999; Hanna *et al.*, 1999; Robertson, 1998; Ross and Fletcher, 1998), provide some promising results for future treatment of breast cancer.

Conclusions

Transgenic mouse model studies have given us insight into possible mechanism of TGF α -initiated breast cancer. Overexpression of TGF α may be an early prognostic factor in the initiation of this disease and in cooperation with secondary transforming events can lead to carcinoma. Importantly, the role of TGF α in the inhibition of apoptosis during normal development could lead to a critical understanding of the role this growth factor plays in normal and neoplastic development in the mammary gland. This growth factor pathway appears to be able to promote growth in the initial phase of neoplastic transformation although it

lacks the dominating transformation ability observed with some of the other EGFR-related mouse models of mammary gland disease like MMTV/erb-2 (Guy *et al.*, 1992; Siegel *et al.*, 1999). In combination with an understanding of TGF α signaling mechanisms, inhibitory agents, which act on the receptor-ligand interaction or on the intracellular kinases stimulated by the EGFR, could lead to practical and efficacious treatment regimens for this disease.

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Signal Transducer and Activator of Transcription 5a Influences Mammary Epithelial Cell Survival and Tumorigenesis¹

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Abstract

The mammary gland undergoes extensive tissue remodeling and cell death at the end of lactation in a process known as involution. We present evidence that the prolactin-activated transcription factor signal transducer and activator of transcription 5a (Stat5a) has a crucial role in the regulation of cell death during mammary gland involution. In a transforming growth factor- α transgenic mouse model that exhibited delayed mammary gland involution, the absence of Stat5a facilitated involution-associated changes in morphology of the gland and the extent and timing of programmed cell death. These Stat5a-dependent changes also affected epidermal growth factor receptor-initiated mammary gland tumorigenesis. Overexpression of the transforming growth factor α transgene in the mammary epithelium reproducibly generated mammary hyperplasia and tumors. In the presence of the activated epidermal growth factor receptor, deletion of Stat5a delayed initial hyperplasia and mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor, and its presence is required for the epithelium of the mammary gland to resist regression and involution-mediated apoptosis. We also suggest that Stat5a is one of the antecedent, locally acting molecules that initiate the process of epithelial regression and reorganization during involution.

Introduction

Involution is the phase of mammary gland development that remodels the molecular and morphological characteristics of the gland after lactation. The systemic levels of prolactin, one of the dominant hormones in the stimulation and maintenance of lactational competence of the gland, decreases at the onset of involution (1). The characteristics of the biolog-

ical activity and regulation of prolactin argue for prominent involvement of this systemic hormone in the induction of mammary gland involution. Prolactin secretion is tightly regulated and is stimulated by suckling. The half-life of prolactin is very short in the blood, and there is evidence that exogenous treatment with prolactin can delay involution at certain stages of development (2, 3). Despite the clear role of this systemic hormone in the initiation of involution, the role of its subservient signal transduction pathways in the regulation of the morphological and transcriptional changes of involution is unknown. The prolactin-activated transcription factor Stat5a³ is essential for the development and differentiation of the mammary gland (4). Stat5a phosphorylation sharply declines within 12 h after weaning, suggesting a role in the onset of involution. In fact, Stat5a has been implicated in the regulation of apoptotic processes in other tissues (5, 6).

Mammary gland involution can be separated into two molecularly and morphologically distinct phases. The first phase is defined by changes in the expression patterns of genes involved in milk synthesis (WAP and ornithine decarboxylase), cell survival and death (Bax, Bcl-2, Bcl-x_s, SGP-2, interleukin-converting enzyme 1, and p53), and proteins that act to regulate potential survival factors (insulin-like growth factor-1, insulin-like growth factor-binding protein 5, and TGF- β) and modulate interactions with the extracellular matrix (tissue inhibitor of metalloproteinase 1; Refs. 3 and 7–11). Importantly, dephosphorylation of the prolactin-dependent transcription factors Stat5a and Stat5b can be detected within 12 h of the initiation of involution (12). Despite the high levels of apoptosis early in involution, the integrity of the alveolar basement membrane is not disrupted. The second phase of involution is characterized by increases in the expression of proteins and genes involved in reorganizing the extracellular matrix (gelatinase 1, stromelysin 1, and urokinase plasminogen activator) and abrogation of milk gene expression. These molecular changes precede the dramatic degradation of the lobuloalveolar acini and the absorption of apoptotic cells. It is presumed that changes in the concentrations of the systemic hormones prolactin, oxytocin, and progesterone, in concert with changes in the activity of locally acting factors, initiate and stimulate the process of involution.

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³ The abbreviations used are: Stat, signal transducer and activator of transcription; TGF, transforming growth factor; Jak, janus kinase; MAPK, mitogen-activated protein kinase; WAP, whey acidic protein; EGFR, epidermal growth factor receptor; BrdUrd, bromodeoxy uridine; WT, wild-type; PCD, programmed cell death; EGF, epidermal growth factor; TBST, Tris buffered saline Tween; TUNEL, terminal deoxynucleotidyl transferase-mediated uridine nick end labeling; ERK, extracellular signal-regulated kinase.

The importance of the Stat proteins in the growth and differentiation of many cell types is exemplified by their ubiquitous expression and widespread utilization in the signal transduction pathways of multiple cytokines (13). Stats are capable of transmitting both differentiative and proliferative signals, depending on the cellular and receptor context (14, 15). In the mammary gland, Stat5a activation by prolactin is required for functional differentiation of the epithelium during pregnancy and lactation (4, 16). Singular tyrosine phosphorylation by the prolactin receptor-associated intracellular kinase Jak-2 is obligatory to maintain Stat5a transcriptional activity (17). However, novel alternative mechanisms of regulating Stat5a activity have been identified. Prolactin-activated signal transduction can tyrosine- and serine-phosphorylate multiple isoforms of the Stat5a molecules (18). Furthermore, truncated forms of Stat5a have transcriptional dominant negative activity (19, 20) and have been linked to the regulation of apoptosis (5). In the liver, EGF can stimulate phosphorylation and nuclear translocation of the Stat5a isoform, Stat5b (21). Importantly, serine phosphorylation of Stat5a by the prolactin-independent, MAPK pathway has been identified, (22) including a direct association between Stat5a and the serine kinase ERK-1/2(MAPK) (23). Serine phosphorylation by the MAPK pathway is required for transcriptional activity of Stat1 and Stat3, but serine phosphorylation is not obligatory for prolactin-dependent transcriptional activity of Stat5 proteins (24). Taken together, these data imply that Stat5a activity can be regulated by the prolactin and EGF signaling pathways and possesses diverse functional roles within a single cell that are dependent on its phosphorylation state and expressed isoforms.

Initiation and progression of breast cancer rely on the inappropriate temporal and qualitative activity of normal cellular signaling pathways (25). These alterations in signaling activity often disrupt the normal mechanisms of mammary gland development. In one mouse model (WAP-TGF- α), overexpression of TGF- α , which binds to and activates the EGFR, delays mammary involution and transforms the mammary epithelium (26). The prolactin signaling pathway has been implicated in loss of normal epithelial differentiation (4, 16) and transformation (27, 28). The role of Stat5a in tumorigenesis is implicated by the appearance of Stat5a overexpression in leukemic (29, 30) and oncogenic (31) transformed cells. Additionally, constitutive activation of the Jak/Stat pathway occurs in multiple leukemia-, v-abl-, and human T-cell lymphotropic virus-transformed T cells (32, 33), and specific inhibition of constitutive Jak-2 activity in acute lymphoblastic leukemia can block cell growth and induce cell death (34). We used the WAP-TGF- α transgenic and Stat5a-null mouse models to explore the *in vivo* role of Stat5a in mammary gland involution and tumorigenesis.

Results

Delayed Mammary Involution in the TGF- α TG Mice Is Curtailed in the Absence of Stat5a. The contribution of Stat5a in regulating involution and the establishment of mammary tumors was investigated in mice that carried the TGF- α transgene (TGF- α TG mice) or were null for the Stat5a gene (Stat5aKO mice) and in Stat5a-null mice that expressed the

transgene (Stat5aKOTGF α mice). H&E-stained mammary glands from TGF- α TG, Stat5aKO, Stat5aKOTGF α , and WT nontransgenic control mice at day 18 of pregnancy and days 1, 3, and 7 of involution were examined by light microscopy (Fig. 1). Morphological differences were observed between the TGF- α TG and Stat5aKOTGF α mammary glands within the first pregnancy and involution. TGF- α TG mammary glands contained hyperproliferative alveoli and ducts. Alveolar lumina contained heterogeneous eosin-positive staining material. A significant increase in the number of stromal cells was evident, in addition to increased deposition of stromal collagen (Fig. 1f). Involution was delayed in the TGF- α TG mice, as reported previously (26). Some cell loss and condensation of lobuloalveolar structures was observed during involution, but the gland lacked the dramatic epithelial regression of the WT gland (Fig. 1, *n* versus *p*). Mammary tissue from pregnant Stat5aKOTGF α mice was histologically similar to pregnant tissue from TGF- α TG mice but contained more epithelium than observed in the glands of Stat5aKO mice (Fig. 1, *a-d*). Secretory structures appeared to be more uniform and lacked the observed heterogeneous precipitations in the alveolar lumens. During involution, the Stat5aKOTGF α gland remodeled more rapidly and extensively than the TGF- α TG gland (Fig. 1, *o* versus *p*). This effect was enhanced after subsequent rounds of pregnancy and involution (data not shown). The primary and secondary ducts of the Stat5aKOTGF α mice lacked concentrations of hyperplastic cells like those found in the TGF- α TG mice. On rare occasions, hyperplastic regions were evident after three or more rounds of pregnancy in Stat5aKOTGF α mice (data not shown).

Apoptosis and Proliferation Levels Are Altered in the Stat5aKOTGF α and TGF- α TG Mice. High levels of apoptosis, decreased proliferation, and the collapse of lobuloalveolar structures in the mammary epithelium are hallmarks of involution in the mammary gland (7). To establish and define the characteristics of mammary involution in the Stat5aKOTGF α and TGF- α TG mice, the levels of apoptosis and proliferation were analyzed by TUNEL and BrdUrd immunohistochemistry, respectively (Tables 1 and 2). WT glands exhibited increased levels of apoptosis within 1 day of the initiation of weaning and exhibited maximal levels at day 3, as expected (9, 35). Consistent with an inhibition or delay in involution in the TGF- α TG gland, the number of apoptotic cells was slightly reduced at day 1 of involution compared with WT and Stat5aKOTGF α glands (Fig. 2A). Apoptosis was reduced in the TGF- α TG gland at day 7 when compared with WT gland at day 7. Whereas Stat5aKOTGF α mammary glands at day 18 of pregnancy ($P = 0.02$) and at day 1 of involution ($P = 0.015$) had significantly higher levels of apoptosis than TGF- α TG and WT mammary glands over the same time period, the levels were similar at days 3 and 7. Interestingly, Stat5aKO mammary glands also displayed significantly high levels of apoptosis at day 18 of pregnancy and day 1 of involution ($P = 0.03$).

Levels of proliferation, as measured by BrdUrd incorporation, were equivalent in mammary tissue of pregnant Stat5aKOTGF α , TGF- α TG, Stat5aKO, and WT mice (Fig. 2B). Generally, 6–10% of the cells in the pregnant gland were

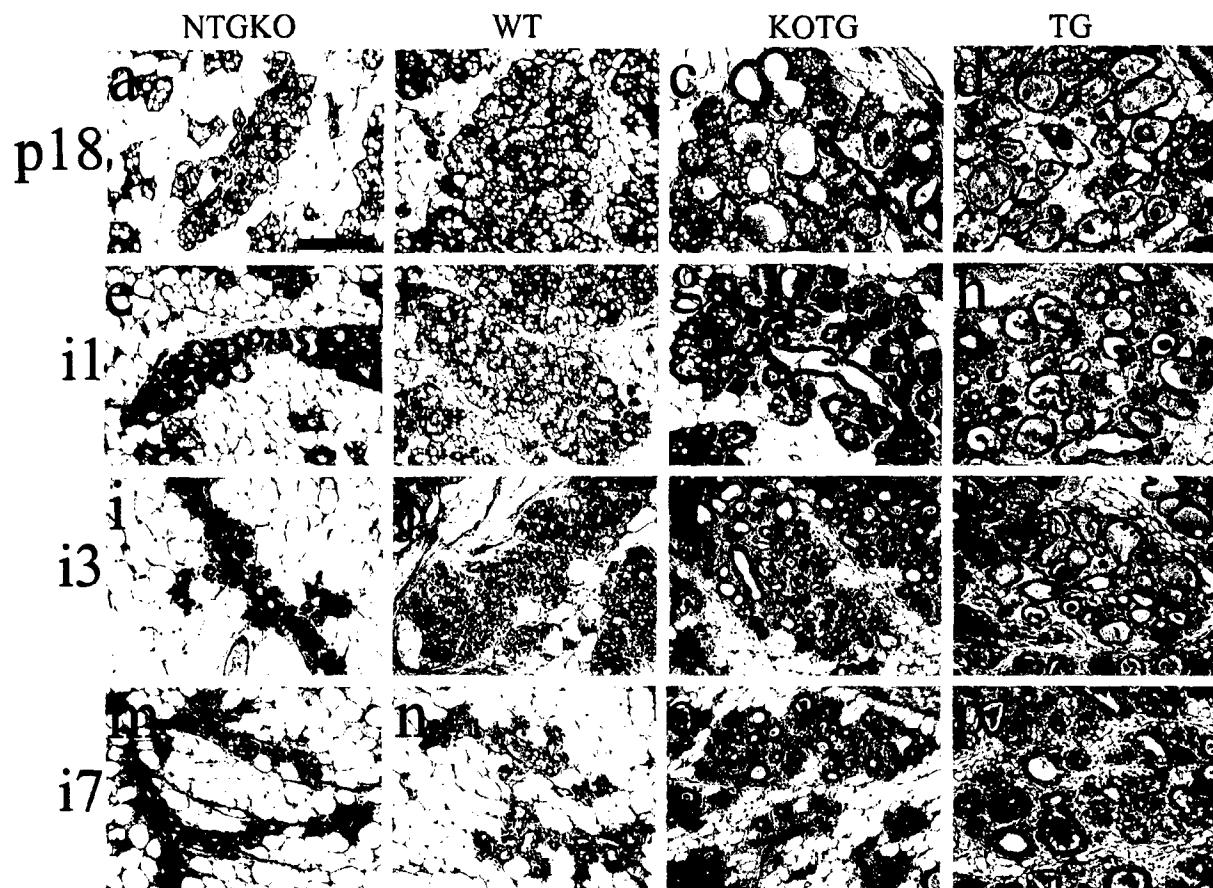


Fig. 1. Involution is enhanced in the absence of *Stat5a*. H&E staining of inguinal mammary glands from *Stat5a*-null nontransgenic (NTGKO), WT, *Stat5a*-null TGF- α transgenic (KOTG), and TGF- α transgenic (TG) mice at 18 days of pregnancy (p18, a–d) and days 1 (i1, e–h), 3 (i3, i–l), and 7 (i7, m–p) of involution. Samples were collected from mice during or immediately after their first pregnancy. Compare epithelial condensation at day 3 and day 7 of involution in the WT (j) versus the KOTG (k) and TG (l). Bar in a, 200 μ m.

Table 1 Percentage of apoptotic cells detected with TUNEL

Values are the mean value \pm SE. Superscript symbols denote mean values that were significantly different from the WT mean value of the same day.

	KOTG	TG	WT	NTGKO
Preg. day 18	4.21 \pm 0.65 ^a	0.86 \pm 0.31	1.47 \pm 0.56	2.61 \pm 0.52
Involution day 1	9.01 \pm 1.46 ^b	2.01 \pm 0.49	3.21 \pm 0.96	7.65 \pm 1.19 ^c
Involution day 3	6.96 \pm 0.41	4.92 \pm 1.92	5.97 \pm 0.75	4.35 \pm 0.14
Involution day 7	3.32 \pm 0.74	1.75 \pm 0.56	4.56 \pm 1.42	4.16 \pm 1.89

^a $P = 0.02$.

^b $P = 0.015$.

^c $P = 0.03$.

Table 2 Percentage of proliferating cells detected with BrdUrd labeling

Values are the mean \pm SE. Superscript symbols denote mean values that were significantly different from the WT mean value of the same day.

	KOTG	TG	WT	NTGKO
Preg. day 18	10.30 \pm 5.16	10.03 \pm 1.77	6.97 \pm 1.18	4.09 \pm 1.72
Involution day 1	7.40 \pm 2.12 ^a	3.63 \pm 1.33	1.38 \pm 0.49	1.53 \pm 0.23
Involution day 3	6.43 \pm 0.64 ^b	4.00 \pm 1.84	0.67 \pm 0.27	0.96 \pm 0.15
Involution day 7	6.87 \pm 0.80 ^c	5.62 \pm 1.05 ^d	0.07 \pm 0.07	0.65 \pm 0.28

^a $P = 0.05$.

^b $P = 0.001$.

^c $P = 0.001$.

^d $P = 0.007$.

labeled with BrdUrd (Fig. 2B). Proliferation was low in involuting tissue from *Stat5a*KO and WT mice (<1%). In contrast, the TGF α TG and *Stat5a*KOTGF α glands had detectable, persistent proliferation levels of 4–7% throughout involution. Apoptotic cells were observed in *Stat5a*KOTGF α mammary glands at day 18 of pregnancy and day 1 of involution in animals labeled exclusively with BrdUrd (Fig. 2C, arrows). The appearance of apoptotic, BrdUrd-labeled cells demon-

strated that DNA synthesis in these cells preceded the initiation of PCD.

WAP-TGF- α Transgene Expression Is Present throughout Mammary Gland Development in *Stat5a*KOTGF α and TGF α TG Mice. The WAP gene promoter has been exploited to target high-level expression of transgenes, including TGF- α , to the mammary epithelium of pregnant mice (36–40). To assure that the hormonal and morphological changes

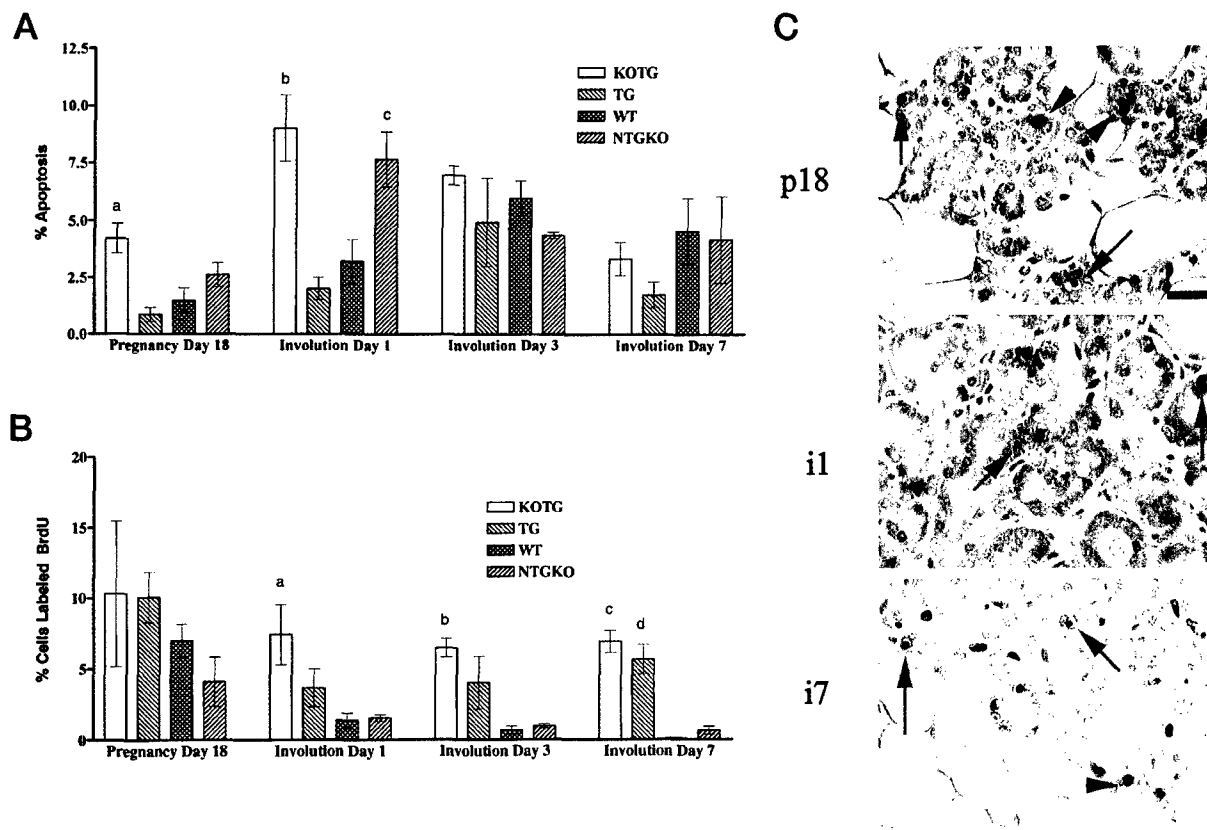


Fig. 2. A, KOTG mammary glands display elevated levels of apoptotic cells during pregnancy and early involution. Apoptosis levels in Stat5a-null TGF- α transgenic (KOTG), TGF- α transgenic (TG), Stat5a-null nontransgenic (NTGKO), and WT mammary glands after analysis by the TUNEL technique. Values represent the percentage of cells (means \pm SE) displaying the label in a minimum population of 1000 cells. Analysis was performed on mice during or immediately after their first pregnancy. Serial sections of tissue from the same mouse were used for the determination of proliferation and apoptosis. A minimum of three mice were analyzed from each genotype (see "Materials and Methods."). Total number of cells counted, 7.5×10^5 . a, b, and c denote significant differences between the KOTG and NTGKO values when compared with apoptosis levels in WT glands at day 18 of pregnancy and day 1 of involution. Specific values for Fig. 2, A and B, are shown in Tables 1 and 2. B, proliferation is higher in TG and KOTG mammary glands through involution. Proliferation levels in Stat5a-null TGF- α transgenic (KOTG), TGF- α transgenic (TG), Stat5a-null nontransgenic (NTGKO), and WT mammary glands after analysis by BrdUrd labeling. Values represent the percentage of cells (means \pm SE) displaying the label in a minimum population of 1000 cells. Analysis was performed on mice during or immediately after their first pregnancy. Serial sections of tissue from the same mouse were used for determination of proliferation and apoptosis. A minimum of three mice were analyzed from each genotype. Total number of cells counted, 6.5×10^5 . a, b, c, and d significant differences between TG and KOTG samples when compared with WT at day 1, 3, and 7 of involution. C, proliferation analysis of mammary gland in Stat5a-null TGF- α transgenic mammary glands. This figure demonstrates the appearance of BrdUrd-labeled apoptotic cells in late pregnancy (p18) and days 1 and 7 of involution (i1 and i7). Mice were injected 2 h before sacrifice with 20 μ g/g body weight BrdUrd, as described in "Materials and Methods." Gray arrows, BrdUrd-labeled apoptotic cells. Black arrowheads, intact BrdUrd-labeled nuclei. Bar in p18, 100 μ m.

associated with involution and the absence of Stat5a did not disrupt transgene expression, TGF- α expression was evaluated in mammary tissue of pregnant and involuting TGF- α TG, WT, and Stat5aKOTGF α mice. Northern blot analysis of TGF- α transgene expression in TGF- α TG and Stat5aKOTGF α mice revealed expression of an appropriately sized transcript at day 18 of pregnancy. Transgene expression persisted through days 1, 3, and 7 of involution in multiple animals (Fig. 3). This expression pattern deviates from the expression of the endogenous WAP gene, which declines at the initiation of involution (41). Endogenous TGF- α expression was only detected in an overloaded lane containing wildtype tissue at day 1 of involution. TGF- α transgene expression persisted through multiple rounds of pregnancy (three to nine rounds) in TGF- α TG and Stat5aKOTGF α mice (data not shown).

The EGFR-activated Serine Threonine Kinase MAPK Remains Active throughout Involution. Binding of TGF- α to the EGFR initiates receptor dimerization and inherent kinase activation, which stimulates the Ras/Raf/Mek signaling cascade. To establish that the EGFR-dependent pathway was being activated by the expression of the TGF- α transgene, Western blot analysis was performed on protein extracts from Stat5aKOTGF α , WT, Stat5aKO, and TGF- α TG animals with an antibody that only recognizes the active (phosphorylated) form of MAPK. Active MAPK was detected in TGF- α TG and Stat5aKOTGF α mammary glands at day 18 of pregnancy and days 1, 3, and 7 of involution (Fig. 4, A and B). Increased levels of the active form of this enzyme were detectable after multiple rounds of pregnancy in the TGF- α TG and Stat5aKOTGF α mammary glands. Very low levels of

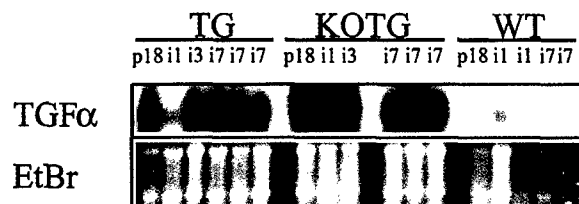


Fig. 3. TGF- α transgene expression persists through involution in KOTG and TG mammary glands. Northern analysis of TGF- α expression in TG, KOTG, and WT mammary glands. Total RNA (20 μ g) was separated and analyzed on a single Northern blot. Each lane represents RNA from a single animal. Samples were collected from mice during or immediately after their first pregnancy. The TGF- α blot was exposed for 4 h at -70°C . EtBr, ethidium bromide staining of RNA.

active MAPK were detected in the nontransgenic Stat5aKO gland (Fig. 4C). Levels were also very low in pregnant and involuting WT mammary glands (Fig. 4D). A significant level of MAPK is present in the lactating, WT mammary gland.

Stat5a Proteins Are Phosphorylated during Involution in TGF α TG and Stat5aKOTG α Mice. Stat5a is phosphorylated on tyrosine 694 by Jak-2, which is associated with the dimerized prolactin receptor (13). Stat5a must remain phosphorylated to maintain its transcriptional activity. One of the earliest molecular events after the onset of involution is the dephosphorylation of Stat5a and Stat5b (42). Immunoprecipitation and Western blot analysis of protein extracts from TGF α TG mice detected high levels of tyrosine-phosphorylated Stat5a and Stat5b at days 1, 3, and 7 of involution (Fig. 5). Conversely, the phosphorylation of Stat5a and Stat5b was decreased at day 1 and remained low to day 7 of involution in WT controls, as reported previously and shown in Fig. 5B (42). Additionally, tyrosine-phosphorylated Stat5a and Stat5b were also detected by immunoprecipitation and Western blot analysis in multiple individual TGF α TG tumors (data not shown). Stat5aKOTG α mammary glands, lacking any Stat5a protein, were assayed for the phosphorylation of Stat5b to determine whether there was a compensation for the absence of Stat5a phosphorylation. A slight increase in Stat5b phosphorylation was detected on days 1, 3, and 7 of involution relative to WT controls.

TGF- α -initiated Mammary Tumorigenesis Is Delayed in Stat5aKOTG α Mice. The rat TGF- α transgene under control of the WAP gene promoter induces mammary tumors in mice bred *ad lib* after three pregnancies (26). The contribution of Stat5a to the establishment of mammary hyperplasias and tumors was investigated in TGF α TG and Stat5aKOTG α mice. Stat5aKOTG α and TGF α TG mice were bred *ad lib* and examined after each round of pregnancy for palpable hyperplasias and tumors. All 48 TGF α TG mice contained palpable hyperplasia in multiple glands by the third pregnancy. TGF α TG mammary glands consistently displayed a swollen appearance by the second pregnancy, emblematic of hypertrophic glands and hyperplasias (data not shown). Gross histological examination revealed the presence of multiple hyperplastic alveolar nodules in multiple glands. TGF α TG glands also contained fluid-filled cysts and stromal proliferation. Some removed tumors were characterized by

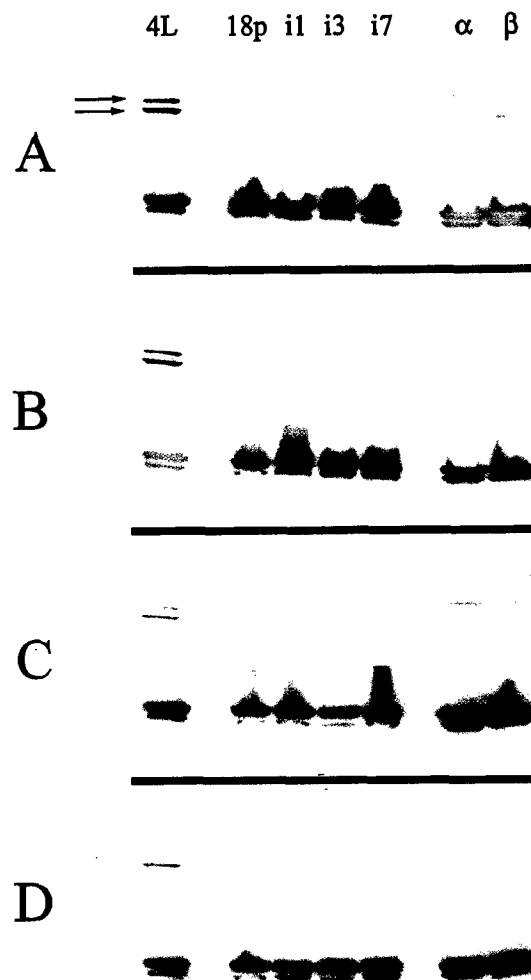


Fig. 4. MAPK is active during pregnancy and involution in TG and KOTG mammary glands. Phosphorylation of MAPK was examined by Western blot analysis with an antibody specific for the active phosphorylated form of MAPK and is shown in the top panel of each section. 18p, i1, i3, and i7 represent samples collected from TG (A) KOTG (B), NTGKO (C), and WT (D) mammary glands. 4L, a control sample from a WT, 4 day lactating mammary gland. α , a sample from a KOTG mammary gland after three pregnancies. β , a TG mammary gland after three pregnancies. Each immunoblot was stripped and reblotted with an antibody to MAPK to determine loading, and the result is shown directly under the phosphorylated MAPK immunoblot in each panel. Arrows in A indicate the two subunits of MAPK, ERK-1 (top) and ERK-2 (bottom).

abnormal hyperproliferation of glandular structures with relatively little hyperplasia. The latency of mammary hyperplasia and tumor appearance and the histology of the hyperplasias and tumors were consistent with the results described previously (26). Hyperplasia and hypertrophy appeared less frequently in the Stat5aKOTG α mammary gland. There was a significant difference in the rate of initial hyperplasia and tumor formation between the TGF α TG and Stat5aKOTG α mice, ($P = 0.003$; Fig. 6). The initial appearance in the Stat5aKOTG α mice was delayed 1.5 months compared with the TGF α TG mice. In Stat5aKOTG α mice

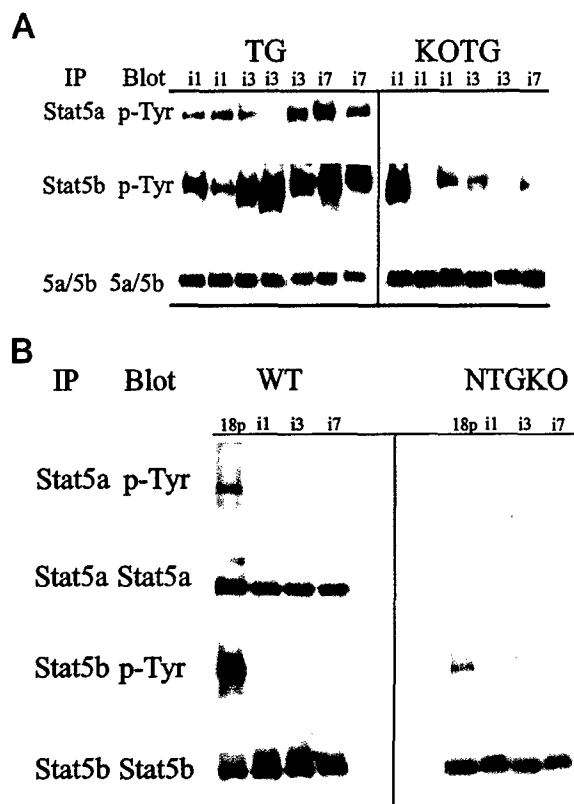


Fig. 5. A, Stat5a remains phosphorylated during involution in TG mammary glands. Stat5a and Stat5b phosphorylation was examined in KOTG and TG mice mammary glands. Protein extracts were immunoprecipitated with anti-Stat5a antibodies (Stat5a, $1:2 \times 10^5$) or anti-Stat5b antibodies (Stat5b, $1:1 \times 10^5$) overnight. Immunoprecipitates were separated by PAGE and immunoblotted with anti-phosphotyrosine (p-Tyr, $1:1 \times 10^3$) antibodies. Samples were collected from mice during or immediately after their first pregnancy. Immunoblots probed initially with p-Tyr were stripped and reblotted with anti-Stat5a antibodies in the TG samples (5a/5b; 5a/5b) or anti-Stat5b antibodies in the KOTG samples (5a/5b; 5a/5b), respectively, to determine protein loading. i1, i3, and i7, days 1, 3, and 7 of involution, respectively. B, Stat5a and Stat5b are dephosphorylated during involution. Stat5a and Stat5b phosphorylation was examined in NTGKO and WT mice mammary glands. Protein extracts were immunoprecipitated with anti-Stat5a antibodies (Stat5a, $1:2 \times 10^5$) or anti-Stat5b antibodies (Stat5b, $1:1 \times 10^5$) overnight. Immunoprecipitates were separated by PAGE and immunoblotted with anti-phosphotyrosine (p-Tyr, $1:1 \times 10^3$) antibodies. Samples were collected from mice during or immediately after their first pregnancy. Immunoblots probed initially with p-Tyr were stripped and reblotted with anti-Stat5a antibodies or anti-Stat5b antibodies to determine protein loading. p18, i1, i3, and i7, day 18 of pregnancy and days 1, 3, and 7 of involution, respectively.

that did develop hyperplasias or tumors, usually only a single gland was involved, as compared with multiple gland involvement in the TGF α TG mice.

Discussion

Loss of Stat5a Activity Is an Early Event in Mouse Mammary Gland Involution. Dephosphorylation and hence inactivation of the transcription factors Stat5a and Stat5b and *de novo* phosphorylation of Stat3 occur in the first phase within several hours of pup removal (42), implicating the Stats and prolactin as early regulatory molecules in the re-

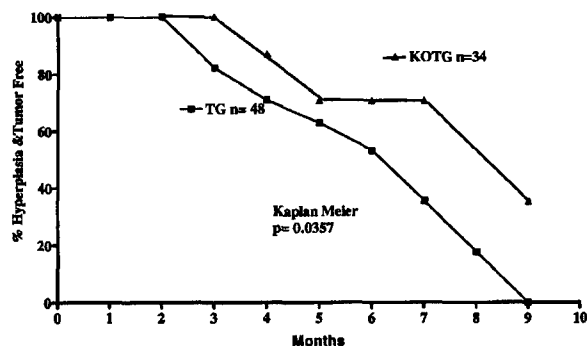


Fig. 6. Absence of Stat5a delays EGFR-mediated hyperplasia and tumor formation. Kaplan-Meier plot of the percentage of hyperplasia and tumor-free KOTG and TG mice over a 9-month time period. All mice were examined at each pregnancy for palpable tumors. There is a significant difference between the two curves ($P = 0.0357$). TG, $n = 48$. KOTG, $n = 34$.

gression of the gland. Using a combination of transgenic and gene deletion mouse models, we have now demonstrated a role for Stat5a in involution that is dependent on its phosphorylation and can be regulated by constitutive EGFR signal transduction. Involution is abrogated on continued activation of the EGFR (and Stat5a) through the expression of a TGF α transgene. In mammary tissue of TGF α TG mice, Stat5a remained phosphorylated, as did Stat5b. However, on deletion of the Stat5a gene, involution proceeded unabated, despite expression of the TGF α transgene. Rapid involution in the absence of Stat5a was accompanied by increased levels of apoptotic cells. Our experiments not only demonstrate that Stat5a is a survival factor *in vivo* but also show that its presence is mandatory to avoid the initiation of PCD in mammary epithelial cells.

Despite the lack of known genetic targets for Stat5a in the involuting gland, there is evidence to suggest that prolactin and Stat5a can control cell survival. Activation of Stat5a has been implicated in the inhibition of apoptosis in T cells through interleukin 2 (6) and interleukin 9 (43) signaling. In addition, selection of apoptosis-resistant immature T cells leads to the activation of a dominant negative form of Stat5a (5). Prolactin can also stimulate cell survival in mammary epithelial cells *in vitro* (44), and prolactin and growth hormone can promote cell survival in the involuting rat mammary gland (2, 3). The presence of apoptotic cells in the mammary tissue of pregnant mice that lack the Stat5a gene and express the TGF α transgene suggests that cell dependence on the presence of phosphorylated Stat5a precedes the actual systemic hormonal switch into involution. This observation also implies that the Stat5a-dependent mechanism of apoptosis induction may be hormonally independent. We did not observe this apoptotic phenomena in the WT and TGF α transgenic mice. This increased number of apoptotic cells during pregnancy was also detected in BrdUrd-labeled Stat5aKOTGTG mice. Analysis of these glands revealed the presence of BrdUrd-labeled apoptotic cells at day 18 of pregnancy and at days 1, 3, and 7 of involution. These cells replicated their DNA and then proceeded directly into PCD.

This association between the cell's ability to replicate its DNA and the progression into the apoptotic program has been reported previously (45). Cells may be programmed to initiate PCD before they enter the cell cycle; consequently, BrdUrd-labeled cells undergoing apoptosis are detected. Interestingly, not all the cells in the Stat5aKOTGF α pregnant gland undergo PCD. This suggests that some cells have obviated the requirement for Stat5a, possibly through an unknown compensatory mechanism. Alternatively, these cells may have a unique differentiation state that precludes them from entering the apoptotic pathway.

Despite persistent phosphorylation of Stat5a in involuting mammary tissue, TGF α TG mice did undergo involution-dependent epithelial reorganization and activation of PCD. This morphological change was characterized by moderate condensation of alveolar structures and loss of cells through the induction of PCD. We propose that the dephosphorylation of Stat5a is not the only guiding factor for apoptosis progression in the involuting mammary gland and that other parallel signals may be required for the complete induction of both phases of involution (12).

Transgenic TGF- α Activates the EGFR and Ensures Persistent Phosphorylation of Stat5a. TGF- α stimulates the EGFR and activates MAPK through the Ras/Raf/Mek signaling cascade. This is exemplified by the presence of the activated form of MAPK during involution in the TGF α TG and Stat5aKOTGF α mice. Persistent activation and phosphorylation of Stat5a in the TGF α TG mice could occur through three distinct mechanisms. The EGFR can directly phosphorylate Stat1 and Stat3 in the absence of Jak activation (46–48). Although direct phosphorylation of Stat5a by the EGFR cannot be ruled out, it was not possible to coimmunoprecipitate this receptor with anti-Stat5a antibodies in the TGF α TG mammary gland (data not shown). Secondly, stimulation of MAPK by EGF can lead to phosphorylation and nuclear translocation of the Stat5a isoform Stat5b in the liver (21). Support for this mechanism comes from results describing that Stat5a and its shorter isoforms can be tyrosine- and serine-phosphorylated by prolactin (18), and Stat5a and Stat5b can be tyrosine- and serine-phosphorylated by prolactin-independent, MAPK-dependent mechanisms (22, 49). A direct interaction was recently demonstrated between MAPK(ERK1/2) and Stat5a in growth hormone-dependent signaling (23). Growth hormone is known to influence mammary gland involution, although this effect is not as dominant as that observed for prolactin (2). Thirdly, EGFR could activate Stat5a through Jak-2. The EGFR is known to use Jak-1 to activate Stat1 and Stat3 (14, 50) and can modulate Stat5 expression in mammary epithelial cells (51). Theoretically, the constitutively active EGFR in the TGF α TG and Stat5aKOTGF α mice could stimulate Jak-2, although this was not formally proven. Alternatively, EGFR could stimulate a secondary Jak, which inappropriately uses Stat5a as a substrate that has been demonstrated in IFN- γ signaling (52, 53). Presumably, the stimulation of the EGFR kinase in the presence of the TGF- α transgene leads to phosphorylation and activation of a downstream kinase, possibly including a Jak, that maintains Stat5a in its phosphorylated state. We suggest that one or more of these mechanisms regulate the

phosphorylation of Stat5a and control apoptosis in the mouse mammary gland.

Persistent Stat5a Activity Contributes to Mouse Mammary Tumorigenesis, and Its Absence Acts to Delay Hyperplastic Epithelial Formation. One of the dominant pathways commonly altered in breast cancer is the EGF signal transduction pathway (25, 54). Gene deletion studies in mice have demonstrated that the EGFR is not dispensable for normal mammary development (55, 56), and that aberrant signaling through this receptor induces neoplastic growth and frank tumors (26, 57–59). Using an EGF-dependent transgenic mouse model, we can now show that the persistent activation of Stat5a is linked to the transformation of the mammary gland. We suggest that the transformation of the mammary epithelium occurs at the expense of a loss of cell survival regulation, which is contingent on the persistent phosphorylation of Stat5a and permits the inappropriate survival of epithelial cells. The appearance of tumors and hyperplasias in Stat5aKOTGF α mice suggests that the absence of Stat5a will not completely block mammary epithelial transformation, and that the TGF- α transgene can activate alternative (non-Stat5a) pathways to bypass PCD and involution and support tumor formation. Such pathways may include Stat5b, which, under specific circumstances, substitutes for Stat5a (60). The involvement of a single gland in a majority of the Stat5aKOTGF α tumors suggests that significant numbers of potentially transformed cells are destroyed during involution. This loss of cells probably contributes to the observed lower rate of tumor formation and the delay in initial tumor appearance. In spite of single gland involvement, survival of the Stat5aKOTGF α mice was no greater than that observed for the TGF α TG mice after initial tumor appearance.

Our findings that Stat5a is a survival factor and that its absence reduces mammary tumorigenesis support findings from other settings. Prolactin and other type 1 cytokines do possess mitogenic activity, implicating the associated Stat molecules with contributing to the mitogenesis of these organs (13). Constitutive Stat activity has been observed in cells transformed by oncogenic viruses (32) and oncogenes (61) and in leukemia cells (29). Moreover, constitutive activation of Jak expression can lead to transformation (27, 62).

Stat5a Is a Survival Factor for the Mammary Epithelium. The absence of Stat5a disrupts the prolactin-mediated survival of the mammary epithelium. Whereas overexpression of TGF- α in the presence of an intact Stat5a results in delayed involution, apoptosis and involution in the absence of Stat5a lead to elimination of the majority of the epithelium. The dephosphorylation or lack of *de novo* phosphorylation and inactivation of Stat5a that occur during involution in the WT gland are mimicked by the absence of Stat5a in the Stat5aKOTGF α mice. Therefore, we propose that the presence of the activated form of this transcription factor prolongs cell survival, and its inactivation or deletion permits PCD to occur. These experiments demonstrate that Stat5a can act as a survival factor for the epithelium of the mammary gland. In addition, they illustrate that the inhibition and/or disruption of the mechanisms that regulate this involution process are critical in mammary gland tumorigenesis.

The conclusions from this study lead us to hypothesize that the inhibition or disruption of Stat5a phosphorylation can lead to protection from transformation in the mammary gland. The application of Stat and/or Jak-specific tyrosine or serine kinase inhibitors may be an approach to blunt the development of mammary tumors originating from EGFR deregulation.

Materials and Methods

Materials. The TGF- α cDNA probe was a kind gift from Dr. David Lee (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC). The generation of the Stat5a antibodies has been described previously (63). Phosphorylated MAPK antibody was purchased from Promega (Madison, WI). Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat antirabbit and rabbit antimouse secondary antibodies were purchased from Transduction Laboratories (Lexington, KY).

Generation of TGF α TG and Stat5aKOTG α Mice. WAP-TGF α TG mice were a gift of Dr. Eric Sandgren (University of Wisconsin, Madison, WI Ref. 26). The generation of the Stat5a-null mice has been described previously (4). TGF α TG mice were interbred with Stat5a-null mice (SvEv129/C57B6) to generate F₁ founders that were hemizygous for Stat5a deletion (Stat5aHT) and transgenic for TGF- α expression. Female Stat5aHTTGF α mice were bred with Stat5aHTTGF α males to generate offspring that were transgenic for TGF- α and homozygous for deletion of the Stat5a gene. These mice were backcrossed for five generations to generate a pure inbred Stat5aKOTG α strain of mice. The same generation TGF α TG and control WT littermates was used for molecular analyses. Confirmation of the presence of the TGF- α transgene was performed by PCR analysis with TGF- α forward primer 5'-TGTCAGGCTCTGGAGAACAGC-3' and reverse primer 5'-CACAGCGAACACCCACGTACC-3'. Stat5a PCR was performed with two sets of primers to knockout (null) and WT alleles: (a) Stat5a forward (5'-CTGGATTGACGTTCTTACCTG-3') and Stat5a reverse (5'-TGGAGTCAACTAGTCTGTCTCT-3') and (b) Neo forward (5'-AGAGGCTATTGCGCTATGACTG-3') and Neo reverse 5'-TTGTCAGATCATCTGATC-3'. PCR for all primers was performed with a denaturing step of 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 56°C for 40 s, and 68°C for 40 s, followed by 10 min at 68°C. Genotype of the mice was confirmed after tissue collection by Northern and Western blot analysis for TGF- α gene expression and absence of Stat5a protein expression, respectively. All animals were housed and handled according to the approved protocol established by the Institutional Animal Care and Use Committee and NIH guidelines.

Mammary Gland Collection. Mammary glands were surgically removed from anesthetized and cervically dislocated mice at day 18 of pregnancy and days 1, 3, and 7 of involution. Day 1 of involution was designated as 24 h after the morning that the pups were born. Pups were immediately removed from the dam after birth and fostered onto a WT mother. The mammary lymph node was removed before homogenization of all glands. Tissues were prepared immediately for RNA and protein extraction, as described previously (42). Whole (number 4) inguinal or (number 3) thoracic mammary glands were surgically excised from mice, spread on Omniset tissue cages (Fisher Scientific, Pittsburgh, PA), fixed for 5 h in Tellyzinkys fixative, and stored in 70% ethanol until processed by standard embedding and sectioning techniques onto Probe-On Plus slides (Fisher Scientific). Sections were stained with H&E.

Immunoprecipitations and Western Blot Analysis. Preparation of protein extracts and immunoprecipitations have been described previously (42). Briefly, 2 mg of fresh and frozen tissue were homogenized in 2 ml of lysis buffer with protease inhibitors; phenylmethylsulfonyl fluoride, leupeptin, and aprotinin at 50 μ g/ml on ice. Protein lysates were rocked for 1 h at 4°C and then cleared by centrifugation at 14,000 \times g for 15 min. The supernatants were removed, mixed with 2 \times loading buffer, and heated to 90°C for 3 min. Samples were spun briefly, electrophoresed under denaturing conditions on 8% precast tris-glycine gels, and transferred to polyvinylidene difluoride membranes according to manufacturer's protocol (Novex, San Diego, CA). Western blot analysis was performed essentially as described with the following exceptions; primary antibody (Stat5a, 1:20,000 dilution; Stat5b, 1:10,000 dilution; anti-phosphotyrosine, 1:5,000 dilution; anti-MAPK, 1:5,000 dilution) was incubated

overnight at 4°C with gentle rocking, and all incubations with antibodies and initial blocking were performed with 3% nonfat dried milk in 1 \times TBST. Detection was performed with the enhanced chemiluminescence kit according to manufacturer's protocol (Amersham) and exposed to Kodak (Rochester, NY) MR autoradiography film. Exposure times are between 1 s and 2 min. Immunoprecipitations with anti-Stat5a antibodies were carried out as described previously (4). Stripping was performed by incubating blots at 56°C in 6.25 mM Tris-HCl (pH 6.8), 2% SDS, and 1% β -mercaptoethanol for 30 min. Blots were washed extensively in TBST and then blocked with 3% nonfat dried milk in TBST.

Northern Blot Analysis. Total RNA was isolated from fresh tissue by homogenization in lysis buffer as described previously (63). RNA was quantified by spectrophotometry and prepared for Northern blot analysis by heating in loading buffer at 65°C. Total RNA was separated by 1.5% agarose gel electrophoresis and transferred to Hybond N+ (Amersham) nylon membrane by capillary transfer with 10 \times SSC. After overnight transfer, the membrane was UV-irradiated and hybridized to each cDNA probe in Quikhyb (Stratagene, La Jolla, CA). Hybridization for the TGF- α cDNA probe was performed in Quikhyb (Stratagene) at 65°C for 16 h, followed by two washes in 1 \times SSC and 0.5% SDS for 30 min, followed by one wash in 0.1 \times SSC and 0.5% SDS for 30 min at 56°C. Blots were hybridized for 16 h and then washed twice in 1 \times SSC and 0.5% SDS for 30 min, followed by one wash in 0.1 \times SSC and 0.5% SDS for 30 min. cDNA and oligo probes were random-primed and Klenow-labeled with [α -³²P]dCTP. After hybridization and washing, blots were exposed from 30 min to 24 h, as specifically described in the figure legends, to Kodak MR autoradiography film at -70°C.

BrdUrd and TUNEL Assays. Protocols for BrdUrd and TUNEL analysis have been described elsewhere (64). Mice were injected 2 h before sacrifice with 20 μ g/g body weight BrdUrd labeling reagent, as described by the manufacturer (Amersham). Each proliferation and apoptosis sample counted represents a minimum of three random fields (at \times 200) and a minimum of 1000 total cells/section for each mouse. A minimum of three mice per timepoint were collected and analyzed. The total number of cells counted for proliferation assay is 6.5×10^5 . The total number of cells counted for the apoptosis assay is 7.5×10^5 . The number of mice collected and the number of mice analyzed are listed by genotype and by timepoints (pregnancy day 18/involution day 1/involution day 3/involution day 7) for apoptosis and proliferation, respectively: KOTG, 4/4/4/3 and 4/3/3/3; TG, 3/5/3/5 and 3/3/3/5; WT, 3/4/5/3/ and 3/3/3/3; NTGKO, 3/3/2/3 and 3/3/3/3.

Hyperplasia and Tumor Analysis. Mice were palpated at the 18th day of pregnancy for mammary hyperplasia, hypertrophy, and tumors. Animals were scored for the presence of either hyperplasia or tumor at every pregnancy. Most animals were sacrificed at the third pregnancy by anesthesia followed by cervical dislocation. In studies that required long-term breeding for tumor development, dams were rebred within 2 days after birth and removal of the pups.

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Abstract to poster presentation at the Breast Cancer think Tank Symposium Held At Chantilly Virginia

Signal Transducer and Activator of Transcription 5a (Stat5a) Regulates Tumorigenesis and Epithelial Cell Survival in the Mouse Mammary Gland

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The mammary gland undergoes extensive tissue remodeling and cell death at the end of lactation in a process known as involution. We present evidence that the prolactin-activated transcription factor, Stat5a has a crucial role in the regulation of cell death during mammary gland involution and can affect the progression of epidermal growth factor (EGF)-dependent mammary tumorigenesis. In a transforming growth factor- α (TGF α) transgenic mouse model, that exhibited delayed mammary gland involution, the absence of Stat5a facilitated involution-associated changes in morphology of the gland and the extent and timing of programmed cell death (PCD). These Stat5a-dependent changes also altered EGF receptor-initiated mammary gland tumorigenesis. Overexpression of the TGF α transgene in the mammary epithelium reproducibly generated mammary hyperplasia and tumors. In the presence of TGF α overexpression, complete genetic deletion of Stat5a reduced the rate of tumor formation and delayed initial mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor and its presence is required for the epithelium of the mammary gland to resist regression and involution-mediated apoptosis. We suggest that Stat5a is one of the antecedent, locally-acting, molecules that initiate the process of epithelial regression and reorganization during involution. In addition, these studies suggest that disruption of the pathway which leads to Stat5a activation can affect the progression of tumorigenesis in the mammary gland. Supported by USMRMC DAMD 17-99-1-9328.

Signal Transducer and Activator of Transcription 5a (STAT5a) Regulates the Survival of Mammary Epithelial Cells and is involved in the Development of Mammary Cancer

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Members of the epidermal growth factor receptor (EGFR) family play a significant role in the initiation and progression of mammary epithelial cell transformation. EGFR stimulation can initiate mitogenic signaling through STAT proteins, particularly Stat5a and Stat3. Previously, we have reported that in the presence of an activated EGFR, deletion of Stat5a from the mammary epithelium, delayed mammary involution by 9 days, and hyperplasia and mammary tumor development by 6 weeks. These observations demonstrate that Stat5a is a survival factor and is involved in delaying mammary tumorigenesis. To evaluate the role of Stat5a and Stat3 in breast tumorigenesis, we examined Stat5a and Stat3 protein expression and activation in breast tumors derived from MMTV-Neu, MMTV-Py-V-MT and MMTV-int3 transgenic mice. We found that the tyrosine phosphorylation level of both Stat5a and Stat3 were elevated in MMTV-Neu breast tumors when compared to MMTV-Py-V-MT and MMTV-int3 tumors. To understand how the EGFR and its downstream kinase signaling pathway contributes to mammary epithelial cell transformation, we used the ErbB kinase inhibitor, AG1478; MAPK kinase (MEK) inhibitor, PD98059; Src kinase inhibitor, PP2; and Jak2/3 kinase inhibitor, AG490 in ErbB2-dependent BT-474, SKBR-3, and MDA-MB-231 human breast cancer cells. Treatment of these cell lines *in vitro* with the kinase inhibitors resulted in reversible G1 arrest in BT-474 and MDA-MB-231 cells. We are utilizing unique combinations of transgenic and knockout mouse models to address the specific contribution of Stat5a and Stat3 in mammary epithelial transformation *in vivo*. We have bred conditional knockout Stat3 null and Stat5a null mice into mice expressing the MMTV-Neu transgene. Together these experiments will allow us to evaluate the contribution of these proteins in the initiation and progression of EGFR dependent mammary tumorigenesis. In addition, through the use of these specific kinase inhibitors, we hope to understand the specific STAT signaling pathways that mediate transformation of the breast. This work will allow development of directed and specific inhibitors of the kinases involved in breast cancer.